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Distribution of Bacterial Cells within Fibrous Air Sterilization Filters

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Summary. Applying radio-isotope ^{32}P, the distribution of bacterial cells retained within glass-fibre air sterilization filters was measured. In particular, the time-dependence of the radial as well as the longitudinal distribution was studied. The collection efficiency of glass-fibre was obtained and the experimental result was compared with a theoretical value based upon the sum of interception, inertia and diffusion. Discrepancies could not be accounted for and are similar to those in the literature. An equation was proposed for the estimation of air filter life. This was not completely verified but leads to some suggestive results.

Introduction

Air sterilization by fibrous filter media is an important auxiliary process to laboratory- or industrial-scale aerobic fermentation.[1, 5]

The removal of bacterial cells by fibrous media has, in principle, many characteristics in common with the removal of small particles of dust or smoke by filtration or by impingement. The theoretical as well as experimental investigations on aerosol filtration have made rapid progress in recent years from the viewpoint of air pollution control.

In parallel with this progress, some studies on bacterial filtration which take into consideration the progress of aerosol filtration have been presented. These deal with the mechanisms of filtration, effects of operating variables on filter efficiencies and the rational design of air filters suited to the particular problem of fermentation.[4, 9, 10, 11]

In this report the distribution of bacterial cells within the fibrous filters and the time-dependence of the distribution were measured by introducing cells labelled with the radio-isotope

phosphorous 32. Despite the recent advances referred to above, not a few problems remain to be solved. The analyses of operating time, i.e. the time during which the filter can be relied upon to retain all the bacterial cells, may constitute one of the most critical problems from the practical viewpoint.

Experimental Apparatus and Procedure

Preparation of Bacterial Cells Labelled by ^{32}P

Test bacteria used in this series of experiments were *Serratia marcescens*. To the culture medium (volume: 15 c.c.) containing glucose, peptone and magnesium sulphate (3, 0·2 and 0·1 per cent, respectively) was added 100 μ of radio-active phosphoric acid. Then the medium was neutralized (pH 7·0) with KOH. After maintaining the culture at 30°C for 20 h, the bacterial cells were centrifuged from the broth, washed three times with distilled water, and suspended in distilled water (15 c.c.) for atomization.

These conditions were kept constant for each preparation. Bacterial cells thus secured were elliptical in form with dimensions $0{\cdot}7 \times 1{\cdot}3\ \mu \pm 0{\cdot}1\ \mu$.

A flow diagram for the experiment is shown in Fig. 1. Some of the air from the air compressor, after being dried by sulphuric acid

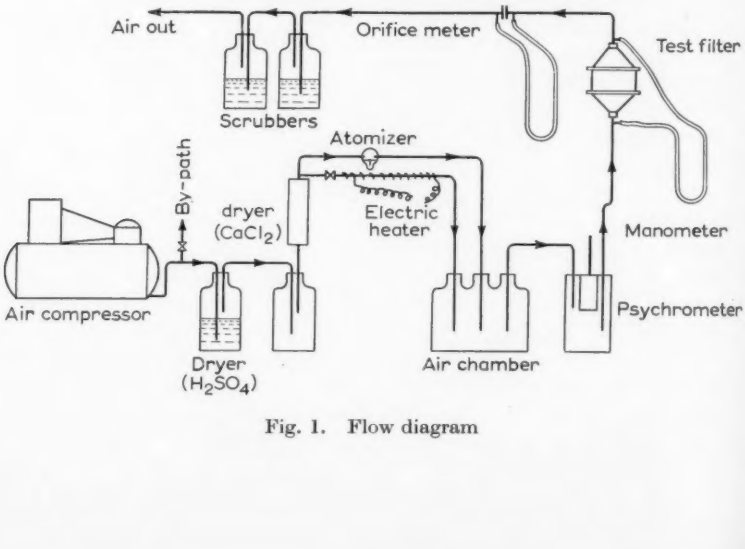

Fig. 1. Flow diagram

and calcium chloride, was used to atomize the liquid bacterial suspension. The other part was heated by the electric heater so that the temperature of the bacteria-bearing air in front of the test filter was always about 25°C. The control of air flow and its measurements were made by the by-pass pipe, the manometer attached to the filter and the orifice flow-meter. The relative humidity of air used was 20–25 per cent. The concentration of bacterial cells in the air before the filter was checked several times by means of a bacterial filter (not shown) and found to be about $10^8/m^3$. The filter material used was glass-fibre of about 13 μ diameter. For ease of handling this fibre was made into six pads, by the Japan Glass Fibre Company, Tokyo, to fit exactly the filter chamber whose diameter and length were 100 and 50 mm respectively. The volume fraction of the fibre within the filter was 3 per cent. The chamber was fabricated from brass and acrylate plastic.

In each experiment a newly prepared bacterial suspension was used, and after a certain period of operation the filter was dismantled to measure the bacterial concentration retained in each pad. After measurement, the filter was reassembled and the experiment continued till the suspension was completely consumed.

Determination of Bacterial Cells Retained in the Filter

The device for measurement of bacterial cells retained in the filter is shown in Fig. 2. After a period of operation the experiment was temporarily suspended and each pad was transferred to a brass holder which was covered by a brass cap as shown in Fig. 2. The brass cap was provided with four holes of 10 mm diameter, each in a particular radial direction. In each determination the radial measurement was made three times by rotating the cap, as shown by the arrow in Fig. 2.

The bell-type GM counter, covered by an aluminium cap, also provided with a hole of 10 mm, was fixed concentrically with each hole in order to count the intensity of β-rays emitted from the bacterial cells. Due precautions were taken during measurement to ensure the stability of the tube and the scaling circuit. The time of counting was adjusted so as to make the error of

measurement around 5 per cent. The intensity of β-radiation was assumed to be proportional to the number concentration of bacterial cells.

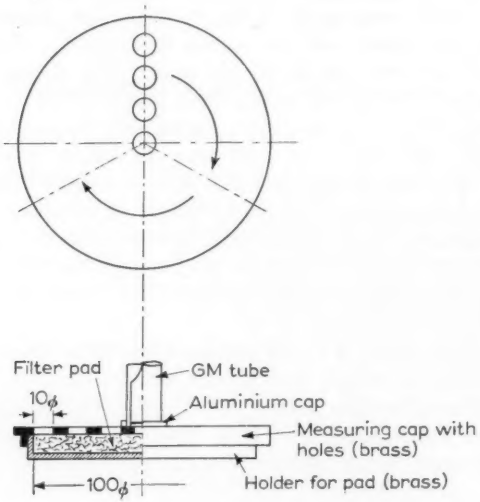

Fig. 2. Determination of distribution of bacterial cells retained in the filter pad

Considering the energy of β-rays of ^{32}P (1·7 MeV) and estimating the range of the β-rays within the filter, it was concluded that the counting rate was approximately proportional to the bacterial concentration within an imaginary cylinder immediately below each hole, whose axial length was equal to the thickness of each pad. Furthermore, wall effects were also checked by placing a filter paper containing a small amount of ^{32}P-labelled bacteria at a distance from the holder to determine the radially distributed intensity. The determination was repeated several times, changing the height of the paper from the base of the holder. No appreciable change in radial or relative intensity was observed. Therefore, so far as such an experimental procedure was concerned the effect of holder wall on the measurement could be neglected.

When the experiment was extended over two days, appropriate calibrations based on the degradation of ^{32}P were made. For convenience, the first day of each experiment was chosen arbitrarily as the datum for calculating intensity.

Experimental Results

Experimental results for the case of $v = 16{\cdot}6$ cm/sec are illustrated in Fig. 3. The ordinates in Fig. 3 (a) to (d) represent the counting rate of β-rays and each is assumed to be proportional to the number concentration of bacterial cells retained in unit volume, as discussed above.

The time-dependence of bacterial distribution in the first pad of glass-wool is shown in (a). The abscissa of (a) shows the radial distance r (mm) from the centre. The first pad is the one first encountered by the contaminated air; it is followed by five other pads, packed successively along the direction of air flow. It is noted from (a) that the radial distribution becomes less uniform the longer the filter is operated. Whether this distribution curve is an indication of the velocity profile of the entering air or whether it corresponds to a difference in the collection efficiencies of glass-fibres remains to be studied.

The rate of accumulation of bacterial cells with time θ (h), so far as the first pad is concerned, is shown in (b), obtained by averaging each curve of (a) with respect to r. Then

$$\bar{N} = C'\theta \qquad \ldots\ (1)$$

C' is a proportionality constant and is considered to be a function of bacterial concentration, velocity of the entering air and so forth. This will be referred to later. The distribution along the depth of the filter is illustrated in (c), relating to $\theta = 4{\cdot}0$ (h). It is interesting to note that the radial distribution gradually becomes uniform as the thickness of filter through which the air passes increases.

By averaging each curve of (c) with respect to r, Fig. 3 (d) with abscissa equal to the filter depth L (mm), is obtained. The curve of (d) is not always continued. Then

$$\bar{N} = \bar{N}_o e^{-\zeta\eta_a L} \qquad \ldots\ (2)$$

where η_a is collection efficiency of glass-fibre whose volume

fraction is α, ζ is experimental constant. This constant is considered to be a function of the porosity, fibre diameter and the depth of filter. This, too, will be discussed later.

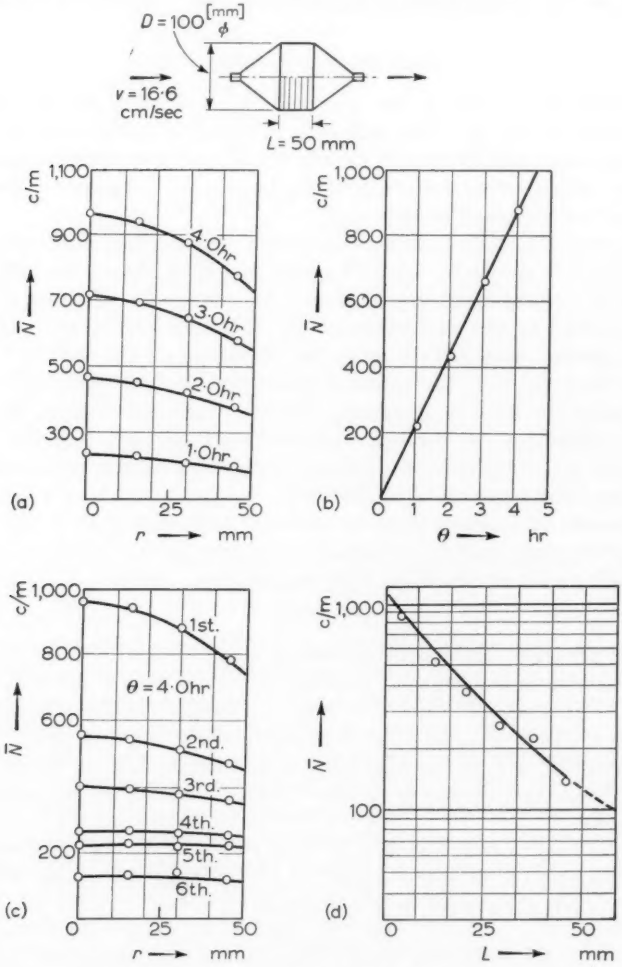

Fig. 3. An illustration of experimental results

In Fig. 4 the retention curve for the case of $v = 8{\cdot}5$ cm/sec is presented, the parameter being θ. Though each curve is not always continued they are near parallel. Therefore, the collection efficiency of glass-fibre is expected to be independent of the operating time θ in a range of experimentation.

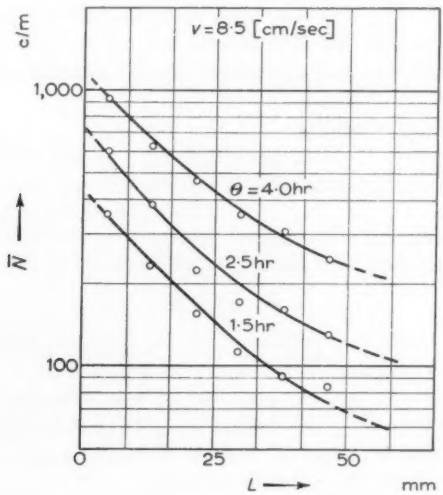

Fig. 4. The retention curve and the operating time

Discussion of Experimental Results

Collection Efficiency

Assuming that the fibre diameter is uniform, the following equation is derived

$$-\ln \frac{N}{N_o} = \frac{4}{\pi} \cdot \eta_a \cdot \frac{1-\epsilon}{\epsilon} \cdot \frac{L}{d_f} \qquad \ldots\ldots(3)$$

where N is number concentration of bacterial cells in the air stream within the filter, N_o is number concentration of bacterial cells upstream of the filter, ϵ is porosity of the filter, and d_f is fibre diameter.

If the retention curve is considered approximately straight within a rather short range of L (the distance through the filter),

$$\eta_\alpha = \frac{N_o'}{N_o} = \frac{N'}{N}$$

Then,

$$-\ln \frac{N'}{N_o'} = \frac{4}{\pi} \cdot \eta_\alpha \cdot \frac{1-\epsilon}{\epsilon} \cdot \frac{L}{d_f} \quad \ldots\ (4)$$

where N', N_o' is number concentration of bacterial cells retained in the filter.

As was already assumed

$$\frac{N'}{N_o'} = \frac{\bar{N}}{\bar{N}_o} \quad \ldots\ (5)$$

The relationship between η_α and η_o, in other words the effect of fibre proximity, has been shown experimentally to be as follows:[2]

$$\eta_\alpha = \eta_o(1 + K\alpha) \quad \ldots\ (6)$$

where η_o is collection efficiency of an isolated fibre, K is an experimental constant ($= 4{\cdot}5$) and α is $0{\cdot}01 - 0{\cdot}10$.

Using equations (4), (5) and (6), η_o was calculated from the experimental results. The values of η_o thus obtained are shown in Fig. 5. The abscissa of Fig. 5 represents the nominal air velocity v (cm/sec), its ordinate being η_o.

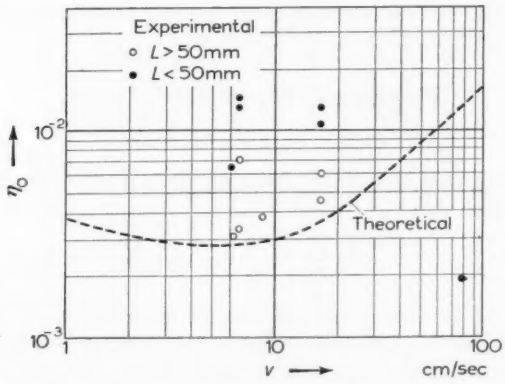

Fig. 5. Collection efficiency of an isolated glass-fibre

In the calculation of η_o, the experimental data for retention were assumed to be composed of two straight lines, the one being drawn in the range below $L = 50$ mm, and the other arbitrarily chosen as the tangential line of the retention curve around $L = 50$ mm. η_o is a function of L and the logarithmic penetration law therefore holds for the shorter layer of filter pad. In this case L was approximately 50 mm.

Theoretically, η_o can be assumed to be the sum of collection efficiency due to inertia η_o', interception η_o'' and diffusion η_o'''.

$$\eta_o = \eta_o' + \eta_o'' + \eta_o''' \quad \ldots\ (7)$$

Since the data for η_o', for the range of N_{Re} in this series of experiments are not available, η_o' was taken from the calculated result published[3] at $N_{Re} = 0{\cdot}2$.

$$N_{Re} = \frac{d_f v \rho}{\mu} \quad \ldots\ (8)$$

where ρ is density of air and μ is viscosity of air. η_o'' and η_o''' were estimated by equations (9) and (10) respectively.[6]

$$\eta_o'' = \frac{1}{2[2{\cdot}00 - \ln N_{Re}]}\left[2(1+R)\ln(1+R) - (1+R) + \frac{1}{1+R}\right] \quad \ldots\ (9)$$

$$\eta_o''' = \frac{1}{2[2{\cdot}00 - \ln N_{Re}]}\left[2\left(1+\frac{2x_o}{d_f}\right)\ln\left(1+\frac{2x_o}{d_f}\right) - \left(1+\frac{2x_o}{d_f}\right) + \frac{1}{1+2x_o/d_f}\right] \quad \ldots\ (10)$$

where R is d_p/d_f, d_p is representative diameter of bacteria, $2x_o/d_f$ is $[1{\cdot}12\ (D_{BM}/C_L d_f)]^{1/3}$, D_{BM} is $CkT'/3\pi\mu d_p$, C_L is $v/2[2{\cdot}00 - \ln N_{Re}]$, C is an empirical correction factor,[8] k is Boltzmann's constant, and T' is absolute temperature.

The result of the calculation is shown by the dotted curve in Fig. 5. The discrepancy between η_o observed and theoretically estimated is apparent and the reasons for this disagreement remain to be investigated. It can be seen that the discrepancies reported by Chen,[2] regarding other conditions of aerosol filtration, are also of the same order of magnitude.

Life of the Air Filter

From the practical viewpoint it is important that the life of a filter, i.e. the period of time during which the filter can be relied upon to retain entering bacterial particles and produce sterilized air, be predictable.

However, the definition of filter life is not simple, particularly from the bacteriological viewpoint. Whether one, or more than one, bacterium emerges from a filter at a time corresponding to the termination of its life, depends upon the particular case under consideration.

In order to simplify the matter, the following assumptions were made.

(1) The collection efficiency η_α is independent of the operating time.

(2) At a time corresponding to the life of the filter, a definite number concentration of bacterial particles N_C' exists at L. N_C' depends upon the particular case under consideration.

(3) From equations (1) and (5)

$$N_o' = C''\theta \qquad \ldots\ (11)$$

C'' depends only upon the bacterial concentration of the air before the filter and the air velocity, being independent of the volume fraction of the fibre and so forth.

Introducing equation (11) into (4),

$$N' = C''\cdot\theta\cdot \exp\left\{-\frac{4}{\pi}\cdot\eta_\alpha\cdot\frac{1-\epsilon}{\epsilon}\cdot\frac{L}{d_f}\right\} \qquad \ldots\ (12)$$

$$N_C' = C''\cdot T\cdot \exp\left\{-\frac{4}{\pi}\cdot\eta_\alpha\cdot\frac{1-\epsilon}{\epsilon}\cdot\frac{L}{d_f}\right\} \qquad \ldots\ (13)$$

where T is life of air filter (h).

Data concerning the life of a glass-fibre filter have been previously presented.[7]

L (mm)	α	T (h)
31·5	0·03	5·0
18·9	0·05	13·5
11·8	0·08	13·5
8·54	0·11	6·0

Filter diameter D = 16 mm
v = 7·5 cm/sec
d_f = 16 μ
T = ±30%

These data were obtained from experiments made parallel to each other by the same procedure. Therefore, because of the constancy of v, C'' in equation (13) was assumed constant. If equation (13) is reasonable, N_C'/C'', i.e.

$$T \cdot \exp\left\{-\frac{4}{\pi} \cdot \eta_\alpha \cdot \frac{1-\epsilon}{\epsilon} \cdot \frac{L}{d_f}\right\}$$

will be kept constant, irrespective of α. Averaging the experimental value of η_o in Fig. 5 and using equation (6), η_α was calculated to estimate N_C'/C''. This result is shown in Fig. 6. The constancy of N_C'/C'' is not apparent.

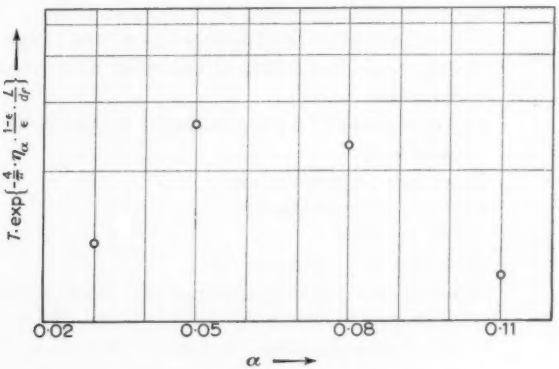

Fig. 6. 'Life' of an air filter

The following facts should be taken into consideration in interpreting the scattered data points.

(1) The experimental error in η_o is about 40 per cent, as shown in Fig. 5. Two-thirds of this deviation will accompany that of N_C'/C'', coupled with an experimental error in T of ± 30 per cent.

(2) The other data points, such as those in Fig. 6, calculated from other life tests, show a scatter of the same order of magnitude, except those in the higher range of volume fraction beyond $\alpha = 0{\cdot}11$. Therefore although the assumptions leading to Fig. 6 were not completely verified, it is proposed that the life of a filter is a worth-while subject for further study.

Nomenclature

C	Empirical correction factor for slip flow
C', C''	Proportionality constants
C_L	$\dfrac{v}{2[2{\cdot}00-\ln N_{Re}]}$
D_{BM}	Diffusion coefficient due to Brownian motion
d_f	Diameter of glass-fibre
d_p	Representative diameter of bacteria, (μ)
k	Boltzmann's constant, erg/degree K
K	Experimental constant
L	Depth of filter
N, N_o	Number concentration of bacterial cells in the air streams within and before the filters, respectively
N', N_o'	Number concentration of bacterial cells retained in the filter
$\bar{N}$, $\bar{N}_o$	Counting rate of β-rays radiated by the radio-active cells, c/m
N_{Re}	Reynolds number of air
R	Interception parameter
T	'Life' of filter
T'	Absolute temperature, °K
v	Nominal air velocity through the filter, cm/sec
x_o	Fluid layer thickness around a cylindrical fibre, across which the particles (bacterial cells) are transferred to the fibre surface due to diffusion, mm
α	Volume fraction of glass-fibre
ϵ	Porosity of the filter
η_α	Collection efficiency of glass-fibre whose volume fraction is α
η_o	Collection efficiency of an isolated single fibre
η_o', η_o'', η_o'''	Collection efficiency of a single fibre due to inertia, interception and diffusion
μ	Viscosity of air, g/cm sec
ρ	Density of air, g/cm^3

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Composition of a Domestic Sewage

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Summary. An analysis of the organic constituents of whole, domestic sewage was made by analysing four physically separated fractions. Settlement, centrifuging and filtration were used to obtain one liquid and three solid fractions. Concentration of the filtrate was achieved by freeze-drying with only minor changes in composition. The average concentration of organic carbon in fresh, whole domestic sewage was 310 p.p.m., of which about 70 per cent was in suspension. Eight classes of compounds were found, on analysis, to comprise about 75 per cent of the organic carbon in whole sewage and of the soluble organic carbon 80 per cent was distributed between five groups. Using chromatographic methods the concentrations of four volatile acids were determined and the presence of other acids was detected. Similarly, seven of the soluble sugars and eight present in hydrolysed suspended solids have been identified. The probable nature of some of the compounds unaccounted for is discussed.

Introduction

It is to be expected that the efficiency of methods of sewage purification could be improved by a better understanding of the biological processes involved and, further, that the study of these processes would in turn be aided by a knowledge of the composition of the organic matter present in the liquid being purified. No comprehensive analysis of sewage seems to have been reported in the literature, however.

Consideration of the origin of sewage suggests classes of compounds likely to be found, e.g. carbohydrates, fats, proteins, urea, and the presence of these has in fact been reported by various authors (e.g. Heukelekian, 1930; Zymny, 1954; Coackley, 1953). Analyses of the various wastes which together constitute sewage are not available in sufficient detail, except in the case of urine, for calculations of expected concentrations of classes of substances to be made with any degree of accuracy.

The present paper describes an investigation made to determine directly the main groups of organic compounds present in a purely domestic sewage and to identify individual members of some of these groups.

Sewage consists of particles of various sizes suspended in a relatively weak solution of organic and inorganic compounds. Because of the difficulty of accurately sampling such suspensions and to minimize changes brought about by biological action, the suspended solids were first removed from the sewage under investigation and analyses were made on the liquid and solids separately. It was convenient to separate the suspended particles in three stages, namely settlement, centrifugation, and ultrafiltration; the coarsest fraction corresponds roughly, in sewage-works practice, to the solids in fresh sewage sludge, which is treated either by anaerobic digestion or merely by physical disposal after drying in open beds. The finer fractions roughly correspond to those solids which, together with the soluble fraction, pass on for aerobic treatment.

Experimental

Materials

Sewage. The details of the sewerage system from which the sewage was collected and some characteristics of the sewage including the inorganic content have already been reported (Painter, 1958). The population served by the sewer was about 6,000 inhabitants of Stevenage New Town and probably contained a higher proportion of children than in the country generally. To minimize the effect of the minute-by-minute fluctuations in the strength of sewage passing along the sewer, 24 h composite samples were collected for analysis. The composite samples were made up of between 25 and 37 sub-samples, each of volume 2·5 l. and each representing 5,000 gallons; the number of sub-samples was dependent on the daily flow of sewage. The results of analysis of three such composite samples, collected on 16–17 July 1957 (No. 1), 23–24 September 1957 (No. 2), and 27–28 May 1958 (No. 3), are reported in this paper.

Preliminary tests showed that the organic carbon and carbohydrate content of sewage fell on standing a few hours at 10° and

20°C, but changes on standing for as long as 24 h. at 0–2°C were insignificant. Consequently all samples were kept at 0–2°C while awaiting fractionation and were maintained at the lowest possible temperature during manipulation.

The pH of the sewage tested varied from 7·7 to 8·4.

Fractionation of sewage. Each 2·5 l. sample was fractionated as described below and the appropriate individual fractions were intimately mixed, before being dried, to form the composite samples.

Settled solids (fraction A). 2·5 l. of sewage was allowed to settle for 1 h. As much as possible of the supernatant liquid was siphoned off and the settled solids were removed from the remainder of the liquid by centrifuging. The solids were then washed with distilled water and re-centrifuged. The washings were rejected.

Centrifuged solids (fraction B). 2 l. of the liquid portion from the settlement were centrifuged for 10 min at 1,200 *g*. The solids were washed with distilled water and re-centrifuged, the washings being rejected.

Candle-filtered solids (fraction C). 1·5 l. of centrifuged sewage was filtered through porcelain candles reputed to remove particles down to 1 μ in diameter. The solids were removed from the candles by means of a fine jet of distilled water and agitation with a 'policeman'. Usually 1·5–2 l. of centrifuged sewage could be passed through a candle having an external surface of about 100 cm^2 before the filtration rate became inconveniently slow. Organic carbon determinations on the solid and on the liquid before and after filtration have shown that the proportion of solids recovered by this method is in the region of 90 per cent.

This fraction and fractions A and B were dried under infrared lamps and ground to fine powders before analysis.

Filtered sewage (fraction D). The first 200 ml or so of the filtrate obtained by passing centrifuged sewage through a candle was rejected, as the content of carbon was found to be lower than that in the bulk of the filtrate. The composite was made by mixing 1 l. portions of each filtrate, all of which were crystal clear and pale or dark yellow in colour. The material was stored at 0–2°C while awaiting analysis.

Freeze-dried sewage. 20 l. batches of composite filtrate have

been concentrated by freeze-drying to buff-coloured powders. This was kindly carried out by the Microbiological Research Establishment, Ministry of Supply, at Porton, and by the Torry Laboratory of the Department of Scientific and Industrial Research. At Porton the material was concentrated in 10 l. batches in the type 3 machine described by Record and Taylor (1958); at Torry the liquid was dried in dishes holding 850 ml each.

Analytical Methods

Dry matter, organic carbon, total nitrogen, ammonia, anionic surface-active agents. These were determined as described in *Methods of Chemical Analysis as applied to Sewage and Sewage Effluents* (Ministry of Housing and Local Government, 1956). The carbon equivalent of any urea present has been subtracted from the organic carbon values reported.

Urea. Determinations of urea *plus* ammonia were made after incubation for 2 h with urease at 30°C.

Organic N. This was calculated by subtracting urea *plus* ammonia from total N.

Pentoses. The method of Mejbaum (1939), as modified by Albaum and Umbreit (1947), was used to determine pentoses. Arabinose was used for preparation of the standard curve.

Hexoses. Hexoses were determined by a method substantially the same as the anthrone method of Trevelyan and Harrison (1952). Glucose was used to prepare standard curves.

Total sugars. Total sugar was determined by the orcinol method of Hewitt (1937); pentoses and hexoses gave approximately the same intensity of colour per mg. This and the two previous tests were applied to filtered sewage and to four extracts or hydrolysates (see below) of each of the three solid fractions of sewage. The values for total sugars in filtered sewage given by this method were in most cases higher by 5–10 per cent than the sum of the hexose and pentose values. The disagreement was even greater in the ethanol and perchloric acid extracts of solids; the orcinol value was as much as 300 per cent greater in some cases. On the other hand agreement was obtained when the tests were applied to HCl and H_2SO_4 hydrolysates of sewage solids. These differences have not been investigated and the

carbohydrate value is reported throughout as the sum of hexose and pentose.

Volatile fatty acids. The double steam distillation method described by Annison (1954) was used to determine volatile fatty acids in liquid and freeze-dried filtered sewage. Liquid samples were first adjusted to pH 8·5 and were then concentrated to small volume *in vacuo*.

Total organic acids and esters. Filtered sewage, adjusted to pH 1·8, was continuously extracted with ether for 24 h, the extract was treated with anhydrous Na_2SO_4 to remove any inorganic acids carried over (Conway and Brady, 1950) and the mixture was filtered. CO_2-free distilled water was added and a two-phase titration was carried out against aqueous 0·01N–NaOH with phenolphthalein as indicator. Control extractions and titrations were made, at the same time, on a solution having roughly the same chloride and phosphate concentration as found in sewage, and the blank value thus obtained was subtracted from the experimental titration. Anionic surface-active agents (largely tetrapropylenyl benzene sulphonate) were extracted and titrated by this procedure but the correction to be made was below 10 per cent of the apparent total acid value, partly because of their high equivalent weight (216 as C) compared with that assumed for water-soluble acids (27 as C, see Table I).

Table I. Assumed proportions of carbon and nitrogen in groups of organic compounds

Group	Substance	Proportion of carbon, %	Proportion of nitrogen, %
Carbohydrates	Glucose	40	0
Amino acids	—	52	16
Higher fatty acids	Stearic	75·5	0
Esters	Tristearin	77	0
Soluble acids	Acetic : Propionic 3 : 1	42	0
Amino sugars	Glucosamine	40	7·8
Anionic surface-active agents	—	56·5*	0
Phenols	Phenol	76·5	0
Sterols	Cholesterol	84	0

* That is, g carbon per 100 g Manoxol O.T. equivalent, in a representative mixture of packaged detergents. The figure therefore contains an allowance for the carbon content of other constituents of packaged detergents.

The solid fractions were extracted with ether in a Soxhlet apparatus for 16 h by the method of Pucher, Wakeman and Vickery (1941). Water-soluble acids were determined on the extract, after treatment with anhydrous Na_2SO_4, by addition of CO_2-free distilled water and titration against aqueous 0·01N–NaOH using phenolphthalein as indicator. Phosphate and chloride were determined in the aqueous phase and an appropriate deduction was made from the acid value.

Higher fatty acids were determined in the separated ether layer of this titration by first evaporating the extract, plus 3–4 ethereal washings of the aqueous layer, to dryness. The residue was dissolved in hot benzene and the acidity of the hot solution was determined by titration with 0·01N-potassium *iso*butylate, using ethanolic phenolphthalein as indicator (Degens, Van der Zee, Kommer and Kamphuis, 1949).

Higher fatty acid plus ester was determined in a similar way after prior hydrolysis of another portion of the solid with 2N–NaOH, followed by acidification, ether extraction and titration. The value for ester was obtained by difference.

Amino acids. Initially the ninhydrin method of Cocking and Yemm (1954) was used to determine amino acids, but later the modification of Rosen (1957) was found more convenient. Glycine was used as a standard. Any ammonia present was first removed by distillation *in vacuo* from a solution adjusted to pH 9·0. The method gave good recoveries when tested on known mixtures of ammonium salts and casamino acids (Difco).

Amide N. This was determined by the method of Bailey (1937).

Amino sugars. These were determined by the method described by Kraan and Muir (1957). Interfering substances were first removed on an ion-exchange resin column, as described by Boas (1953). Glucosamine was used to prepare standard curves.

Creatinine. The Jaffé reaction was used, as described by Hawk, Oser and Summerson (1954).

Sterols. Free and esterified sterols were determined by the Liebermann–Burchard reaction as described by Peters and Van Slyke (1932). Cholesterol was used as the standard.

Phenols. Free and conjugated phenols, containing an unsubstituted *para* position, were determined by steam distillation of the acidified sample and application of the 4-amino-antipyrine

test (Dannis, 1951) to the distillate. Results are expressed in terms of phenol.

Method of expressing results. As the total weight of organic matter present in sewage was not accurately known, it was considered that the best way of expressing the results would be as organic carbon. The proportion of carbon in the various classes was calculated on the assumption, thought not to involve too large an error, that each group consisted of one or two known substances (Table I).

Extractions and Hydrolyses

Carbohydrates. Carbohydrates of increasing molecular complexity were extracted from fractions A, B and C in the following way:

(a) The solid was repeatedly extracted with hot 80 per cent (V/V) ethanol until the anthrone reaction of the extract was negative. This procedure extracts low molecular-weight sugars (Bell, 1955). Separation of the solid was effected by centrifuging.

(b) The residue from (a) was extracted with perchloric acid as described by McCready, Guggolz, Silviera and Owens (1950) and the solid was again separated by centrifuging. This procedure extracts starches.

(c) The residue from (b) was refluxed with 0·25N–HCl for 1 h to hydrolyse polysaccharides to monosaccharides. Centrifugation was again used to separate the solid.

(d) Undissolved cellulose and other polysaccharides were brought into solution by treating the residue from (c) with 72 per cent (V/V) H_2SO_4 for 2 h in the cold and refluxing the mixture, diluted to 1 per cent (V/V) H_2SO_4, for 16 h.

Bound amino acids. The solid (100 mg) was heated with 30 ml 6N–HCl in a sealed tube at 100°C for 8 h (Cummins and Harris, 1956) or refluxed with 100 ml 6N–HCl for 16 h. HCl was partially removed by repeated evaporation from the hydrolysate which was then neutralized before analysis by the ninhydrin method. The two methods of hydrolysis gave almost identical results.

Amino sugars. Solids (100 mg) were hydrolysed under various conditions but it was found that the maximum yield of amino sugar resulted after 12 h refluxing in 100 ml 2N–HCl.

Chromatography

Acids. Volatile acids were concentrated by evaporating the neutralized second steam distillate (see Volatile fatty acids, above) to dryness on a steam bath (Annison, 1954). The acids were converted to their ammonium salts which were then separated by one-dimensional descending paper chromatography using *n*-butanol–1·5N ammonia (1 : 1) (Duncan and Porteous, 1953). Dr. E. F. Annison kindly analysed some samples by gas-chromatography (Annison, 1954).

Volatile and non-volatile acids have been examined by silica-gel column chromatography using chloroform-*n*-butanol mixtures as the mobile phase and the general method of Bulen, Varner and Burrell (1952).

Carbohydrates. Three methods were used for preparing the samples for analysis for sugars. Subsequent analysis showed that the number and position of the individual sugars was the same for a given sample whichever of the three methods was used, so that the first method described, being simpler, was used more than the others.

(1) The hydrolysate (whole solid refluxed 1 h with 0·25N–HCl, or heated at 100°C in sealed tube for 1 h with N–H_2SO_4) was neutralized and evaporated to dryness on the steam bath. Sugars were extracted from this residue, or directly from the freeze-dried powder in the case of soluble sugars, by treatment with pyridine below 40°C (McGeown and Malpress, 1952). The filtered extract was evaporated to dryness *in vacuo* below 40°C and the solid so obtained was dissolved in a small volume of 10 per cent *iso*propanol.

(2) Salts and amino acids were removed from the hydrolysate (or from untreated filtered sewage or a solution of freeze-dried powder) by passage through ion-exchange resin columns (Amberlite IR–120H and IRA–400, CO_3^{--} form. Phillips and Pollard, 1953). The purified sugar solution was concentrated to small volume *in vacuo*.

(3) The hydrolysate was de-proteinized, freed from amino acids and peptides, and concentrated by freeze-drying, as described by Gottschalk and Ada (1956).

In each case 5–25 μl., but usually 10 μl., were taken for analysis

by paper chromatography. The chromatograms were developed by the descending technique for two periods of 20 h, the papers being dried between each development. Various solvent systems were tried. Those giving best separations were *n*-butanol–acetic acid–water (4:1:5) (Gottschalk, 1955), ethyl acetate–pyridine–water (2:1:2) (Jermyn and Isherwood, 1949), *n*-butanol–pyridine–water (45:25:40) and phenol saturated with water (Block, Durrum and Zweig, 1955). The sprays used to detect the various sugars were *p*-anisidine hydrochloride, aniline hydrogen phthalate, naphthoresorcinol (Block, Durrum and Zweig, 1955) and urea (Isherwood, 1954).

An approximately quantitative determination of the major anthrone-reacting sugars present in fraction D was made by applying the anthrone test to eluates of appropriate strips cut from thoroughly dried chromatograms and comparing the colours formed with appropriate standards.

Results

Main Gross Components of Domestic Sewage

An estimate of the contribution made by each gross component of sewage to the content of organic material is given in Table II.

Table II. Amounts of carbon, nitrogen, and ammonia in the main gross components of sewage

(Values given are g per person per day, except faeces and urine which are g per adult per day. Figures in brackets are ranges)

Component	Organic carbon excluding urea	Organic nitrogen	Ammonia plus urea (as N)
Faeces	17 (12–22)	1·5 (1–2)	—
Urine	5	1·7 (0·5–3)	10·5 (7·5–17)
Dish-washing and food-preparation waste water	8	0·2	0
Personal and clothes washing	7	—	0
Total	37	3·4	10·5 (7·5–17)
Whole sewage (average of three composites)	38	3·4	5·3

The values have been taken from the literature, or have been determined on a small number of samples of the component. The data given are admittedly only approximate (agreement between calculated and observed values is considered fortuitous) but serve to show that although the bulk of the organic nitrogen is derived from excreta, only about 60 per cent of the organic carbon comes from this source.

Distribution of Carbon and Nitrogen Between Physically Separated Fractions

Table III shows the distribution of organic carbon and nitrogen between the three grades of suspended solids and soluble matter for three composite samples of sewage. The concentration of carbon in the three samples was relatively constant in spite of the difficulty of sampling whole sewage accurately. There was some variation between samples in the manner in which organic matter was distributed but on average the coarsest solids formed the largest proportion of total organic carbon with the soluble fraction contributing only slightly less. The largest proportion of organic nitrogen was usually formed by the soluble matter.

Table III. Distribution of organic carbon and organic nitrogen in sewage between suspended solids and solution

(Figures in brackets are p.p.m.)

Component	Organic carbon, % of total		Organic nitrogen, % of total	
	Mean of 3 determinations	Range	Mean of 3 determinations	Range
Settled solids	34	27–39	23	18–27
Centrifuged solids	22	16–26	20	15–25
Candle-filtered solids	15	10–22	20	11–33
Candle filtrate	29	26–34	37	28–51
Whole sewage	100 (310)	— (300–320)	100 (27)	— (24–28)

Composition of Soluble Matter

A general analysis of candle-filtered sewage (fraction D) is given in Table IV and shows that the values for the three composites did not vary greatly about the mean. Only about one-quarter of the solids in solution was organic (assuming the C content to be 40 per cent) and organic nitrogen comprised about one-fifth of the total nitrogen. The organic carbon : organic nitrogen ratio averaged 9·0.

Table IV. Composition of candle-filtered sewage and of derived freeze-dried solid

General data

Constituent	Concentration in liquid, p.p.m.		Recovery on freeze drying %	
	Mean of 3 determinations	Range	Mean of 3 determinations	Range
Total solids	791	688–874	73	70–77
Urea (as N)	12	10– 16	53	44–61
Ammonia (as N)	31	28– 32	16	2–23
Organic nitrogen	10	7– 14	92	82–99
Organic carbon	90	82–106	67	63–71

Organic constituents

Concentration (carbon as % of total carbon)

	In liquid		In freeze-dried solid	
Carbohydrate	31·3	25·6–37·8	29·5	13·6–48·2
Volatile acids	11·3	4·7–19·5	4·6	1·2– 7·5
Non-volatile acids	15·2	11·3–18·2	12·7	8·2–19·5
Free amino acids	3·1	2·4– 4·6	4·0	1·3– 6·8
Bound amino acids	7·6	7·3– 8·3	6·8	5·2– 7·9
Anionic surface-active agents	11·2	8·5–14·6	12·5	9·6–16·6
Total C accounted for	79·8	76·8–84·4	70·1	59·6–85·3

The concentration of organic groups has been expressed as percentage of organic carbon in the sample to give a better basis for comparison between the three composites. Variation between

corresponding values was reasonably small except for those for volatile fatty acids. About 80 per cent of the total carbon was accounted for in six groups and in each sample carbohydrates formed the largest single group. The ratio of hexose to pentose ranged from 10 to 12. Free amino acids and low molecular weight peptides were regularly present in lowest concentration of the six groups determined.

It is evident (Table IV) that there was some mechanical loss on freeze drying, apart from loss of ammonia by volatilization. The organic content of the freeze-dried product did not exactly reflect that of the filtered sewage from which it was prepared. This was due partly to preferential loss of volatile acids and, probably, partly to changes occurring in the liquid during transit to the Station where the concentration was carried out. However, apart from the differences in content of volatile acids, the analysis of the freeze-dried solid was in fair agreement with that of the liquid. The solid was also a convenient form of the material for use as a starting point for some chromatographic analyses and in which to test for substances thought to be present in such low concentration that the usual tests were not sufficiently sensitive to apply to unconcentrated sewage. In this way a further 3·5 per cent of the organic carbon has been allocated, in samples 2 and 3, to creatinine (3·2 per cent) and phenols (0·3 per cent).

Of the nitrogen present in samples 2 and 3, creatinine and free and bound amino acids accounted for an average of only 52 per cent.

Composition of Suspended Solids

A general analysis of the three grades of suspended solids showed that the content of organic nitrogen increased and the carbon : nitrogen ratio decreased with decreasing particle size. The concentration of organic groups expressed as percentage carbon of total carbon in the sample are given in Table V. As with soluble matter, variations about the means were on the whole reasonably small. About 63 per cent of the organic carbon in fractions A and B, and rather more in fraction C, were accounted for in seven groups. Higher fatty acids formed the largest group in all fractions and comprised between one-quarter and one-third

of the carbon in each fraction. A large contribution came from esters, especially in fractions A and B. Bound amino acids made up the next largest group and, reflecting the higher carbon : nitrogen ratio, fraction C contained more of this group than did the coarser fractions. On the other hand, there was a larger proportion of carbohydrates in the coarsest fraction than in the others. Most carbohydrate was extracted from all fractions by perchloric or hydrochloric acids and least by ethanol or sulphuric acid. The ratio of total hexose : total pentose was fairly constant with a range of 2·0–2·6 for the three fractions, though the ratio for a given extractant varied with the fraction.

Table V. Composition of suspended solids from sewage
(Figures in brackets are the ratios of hexose to pentose)

Constituent	Concentration, expressed as carbon of total carbon, %					
	Settled solids, A		Centrifuged solids, B		Candle-filtered solids, C	
	Mean of 3 determinations	Range	Mean of 3 determinations	Range	Sample 2	Sample 3
Carbohydrates successively extracted by						
EtOH	1·9 (5·4)	1·5– 2·7	0·4 (1·4)	0 – 0·7	1·5 (1·5)	0·5 (1·4)
$HClO_4$	3·4 (2·2)	1·7– 4·5	1·4 (1·5)	0·6– 2·2	2·1 (1·4)	2·8 (1·5)
HCl	2·9 (2·0)	2·4– 3·3	1·6 (3·3)	1·2– 2·5	2·1 (3·5)	1·0 (5·0)
H_2SO_4	0·7 (2·5)	0·2– 1·6	0·3 (3·8)	0·1– 0·8	0·2 (3·0)	0·6 (5·0)
total	8·9 (2·6)	6·8–10·9	3·7 (2·1)	2·5– 4·8	5·9 (2·0)	4·9 (2·1)
Amino acids—combined	9·5	7·3–11·5	9·9	8·2–11·8	17·1	13·0
Acids—insoluble	25·5	11·5–35	31·9	25·0–38·6	33·8	35·8
soluble	2·0	1·4– 2·4	3·2	2·5– 4·0	1·7	1·9
Esters	15·8	11·5–20·8	13·4	12·2–14·0	9·6	10·6
Anionic surface-active agents	1·3	0·5– 2·3	1·4	0·8– 1·8	3·2	1·8
Amino sugars	0·3	0·3– 0·3	0·2	0·1– 0·4	1·2	0·4
Total accounted for	63·3	45 –74·6	63·7	57·2–71·3	72·2	68·4

Free and esterified sterols were present in each of the three fractions from experiment 3 to the extent of 0·6–1·5 per cent expressed as proportion of total carbon in the respective solids.

Amide N was determined in samples 2 and 3 only. Including

these values the proportion of total organic nitrogen accounted for in fraction A was 59 per cent, in fraction B 44 per cent and fraction C 54 per cent (average of samples 2 and 3).

Composition of Whole Sewage

The full analysis of whole sewage, based on results given above, is presented in Table VI; only two sets of results are included as fraction C of sample 1 was not collected. The results show that each organic group was divided between solution and suspended solids in much the same way in both samples.

Table VI. Composition of whole sewage

Constituent	Concentration in whole sewage (p.p.m. carbon)					
	Sample 2			Sample 3		
	In solution	In suspension	Total	In solution	In suspension	Total
Carbohydrates—total	21	12	33	40	15	55
Amino acids—free	2	0	2	5	0	5
—bound	6	19·5	25·5	8	23	31
Higher fatty acids	0	74	74	0	71	71
Soluble acids	28·5	6·0	34·5	17	4	21
Esters	0	37·2	37·2	0	28·2	28·2
Anionic surface-active agents	12	5	17	11	3	14
Amino sugars	0	0·9	0·9	0	0·5	0·5
Amide	0	1·2	1·2	0	1·5	1·5
Creatinine	2·7	0	2·7	3·5	0	3·5
Total—by analysis	82	218	300	106	205	311
—by addition	72·2	155·8	228	84·5	146·2	230·7
Proportion accounted for, %	88·0	71·4	76·0	79·7	71·3	74·2

Sugars

The sugars identified chromatographically in solution and in hydrolysed solid fractions are listed in Table VII. In solution the predominant sugar was either glucose or sucrose, with lactose, galactose and fructose present in smaller amounts. Sucrose was

usually the main component in snap samples of sewage collected between 8 and 9 a.m. The presence of the disaccharides was confirmed by the disappearance of the spots due to those sugars and by the increase in intensity of the spots due to glucose, galactose and fructose on chromatograms of hydrolysed filtered sewage. Xylose and arabinose were the only pentoses positively identified in solution; ribose was detected only in fractions B and C and the methyl pentose, fucose, was detected only in C. Glucose was the main component in the hydrolysates of all three solid fractions.

Table VII. Individual sugars present in filtered sewage and hydrolysed suspended solids

(The symbols give only a very rough indication of the proportion present. Numerical values under 'candle filtrate' are percentages of anthrone-reacting sugars pipetted on to paper chromatogram)

Sugar	Hydrolysed settled solids (A)	Hydrolysed centrifuged solids (B)	Hydrolysed candle-filtered solids (C)	Candle filtrate sample 1	Candle filtrate sample 2
Glucose	+++	+++	+++	51	16
Galactose	++	+	+	9	<3
Mannose	trace	trace	–	–	–
Lactose	–	–	–	13	17
Sucrose	–	–	–	16	63
Maltose	trace	–	–	–	–
Arabinose	++	+	trace	+	+
Xylose	+	+	+	+	+
Ribose	–	trace	+	–	–
Rhamnose	+	+	++	–	–
Fucose	–	–	trace	–	–
Fructose	–	–	–	++	+
Total anthrone reacting	–	–	–	89	96

There were also a further six or more unidentified substances, present in small concentration, which reacted with *p*-anisidine or

aniline hydrogen phthalate. Some of these substances in the hydrolysates may have been low molecular weight polysaccharides formed during hydrolysis.

Acids

Four steam-volatile acids were detected in filtered sewage by the three chromatographic procedures described above. The acids behaved as acetic, propionic, butyric and valeric but it was not possible to distinguish which isomers of the last two were present. The concentrations of these acids in sample 2 were determined by the silica–gel–column method (Table VIII). In snap samples of sewage, analysed by the gas chromatographic method by Dr. E. F. Annison, the ratios of the acids to one another have been similar to those found for sample 2.

Table VIII. Concentrations of volatile acids in sewage by silica gel chromatography: comparison with published data

Recovery of total volatile acids in present experiment = 88%

Steam volatile acid	Concentration in sewage, p.p.m.		
	Candle filtrate (sample 2 present work)	American whole sewage (Mueller *et al.*, 1958)	
		Sample 'a'	Sample 'b'
Acetic	10·0	11·0	2·5
Propionic	2·6	2·8	2·2
Butyric	1·0	1·0	1·4
Valeric	0·4	not found	

A number of non-volatile acids have been detected by the silica–gel–column method. Citric and lactic acids appear to be present but the nature of the others has not yet been determined.

Discussion

From Table VI it is seen that the total of the compounds estimated accounts for about 75 per cent of the organic carbon in whole sewage; the corresponding proportion for organic nitrogen

was about 50 per cent. These proportions have been calculated using values for the carbon and nitrogen content of the groups which admittedly are not accurate but which are thought to be close to the actual values: also, some of the methods of determination employed, although tested with mixtures of known substances and found to give reasonably reliable results, could perhaps be improved upon. However, the methods of determination and calculation were sufficiently accurate for the present purpose. It is to be emphasized that the analyses refer to fresh sewage from a group of about 6,000 people. The composition may be expected to be different in sewage which is not so fresh and even domestic sewage from other sources may have a somewhat different composition, depending on local habits.

As much as 80 per cent of the organic carbon in solution has been accounted for and it seems unlikely that any major component in solution has been overlooked; this is not so in the case of the solid fractions.

Some of the carbon unaccounted for may be attributed to the additional classes of compound which have been detected in sewage by various workers. These are present in only minute amounts (0·001–1 p.p.m.) and have usually been determined by biological, microbiological or chromatographic methods. Such compounds include vitamins, hormones, β-indolylacetic acid, indole, skatole and condensed aromatic hydrocarbons. Pigments, waxes and lignin would be expected to be present in more than trace amounts, especially in suspension. Indeed, Heukelekian (1930), using the non-specific 72 per cent H_2SO_4 method, found 5 per cent lignin (of dry weight) in fresh sludge and in one determination of the methoxyl group in fraction A in the present work this value was confirmed.

Some of the difference between nitrogen found by analysis and by addition of estimated compounds may be attributed to uric acid, hippuric acid and other nitrogen-containing urine constituents not included in the present analysis. Since these are soluble there remains the problem of the undetected insoluble compounds. In analyses of sewage sludge the Kjeldahl–nitrogen figure is usually taken to be protein but the validity of this assumption does not seem to have been verified. The difficulties of accounting quantitatively for all the amino acids present in

proteins in vegetable matter by hydrolysis without previous separation of the protein have been reviewed by Pirie (1956). Various conditions of hydrolysis of sewage solids have been used in this work, including those thought by Pirie to give minimum losses of amino acids, yet only about 45 per cent of the Kjeldahl–nitrogen reacted in the ninhydrin test. The modified test used was claimed by Cocking and Yemm (1954) to give nearly equal colours per mg nitrogen with most amino acids. This was confirmed in that, when applied to hydrolysates of casein, fibrin and gliadin, values were obtained which, together with the amide nitrogen present, were in good agreement with total nitrogen in the proteins. However, hydrolysates of sewage solids, contained, besides humin nitrogen, soluble nitrogen, which did not react as amino acid or ammonia. Further work is clearly necessary to establish whether the bound amino acid values reported here are low or whether other nitrogen-containing compounds are present in suspension.

The values reported for the concentrations of acetic, propionic and butyric acids in Stevenage sewage are of the same order as those found in snap samples of sewage from an American city by Mueller, Larsen and Lennarz (1958) (Table VIII), using a similar technique. The American sewage also contained formic and lactic acids but not valeric acid; the organic carbon content of the sewage was not reported.

The average value of 8·9 per cent (as glucose in dry solid) for carbohydrates in fraction A agrees well with published figures for fresh sludge; thus, Heukelekian (1930) found 9 per cent and Coackley (1953) reported 11 per cent. No reports appear to have been published on the identity of the sugars present in sewage.

Also present on the sugar chromatograms were small amounts of fluorescent compounds, detected by viewing under ultraviolet light, but they were not identified.

The expected concentration of higher fatty acids has been calculated from the annual sales of soap and from published data on the normal fat content (as free acid and salts) of faeces. Assuming none remains in solution, 30–43 per cent of the total carbon in suspension should be stearic acid carbon; this compares well with determined values of 34 per cent (sample 2) and 34·5 per cent (sample 3).

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Agitation Effects in Yeast Propagation

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Summary. The influence of mechanical agitation in aerobic fermentations is felt mainly through increased aeration efficiency. For yeast propagations in which oxygen supply is abundant—and presumably not limiting to growth—a specific, but limited, effect of fluid agitation on growth rate has been demonstrated. The improved growth rate is attributed primarily to better mixing of the entire fluid mixture rather than to improved cell-fluid mass transfer.

Introduction

The success of submerged fermentation processes depends in great measure on adequate aeration and agitation, and a great deal of attention has been given to this composite problem. So far, however, quantitative examination has largely been limited to absorption of oxygen from the sparging gas. Agitation effects—independent of aeration—remain virtually unstudied.

Agitation may, in fact, influence many other aspects of the cell-substrate interaction besides oxygen transfer. Many people have commented on these specific effects of agitation but experimental evidence is largely indirect. One example is the work of Bartholemew *et al.*,[1] demonstrating the need for a minimum agitation power input in streptomycin and penicillin fermentations if oxygen transfer from medium to cell is not to be limiting. Others have noted favourable effects of mechanical and inert-gas agitation in anaerobic fermentations.[3, 8]

In all fermentations, bulk turnover and complete mixing of the fermenter contents is essential. This is a matter of fluid

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throughput, with the agitator acting like a pump, and is related to power input and the design of the agitation system.

Another aspect of agitation is encountered in the case of fermentations involving heavy growths of filamentous organisms. These mycelial masses apparently must be broken down—at least in part—if the fermentation system is to behave as a reasonably homogeneous reaction mass. An extension of this is the possibility of improving the propagation rates of seed cultures by partial fragmentation. If the 'tips' of hyphae are more active in growth, then partial fragmentation, resulting in more of these growth sites, should enhance inoculum development, as claimed by Vander Brook and Savage.[9]

The variety of agitator designs proposed for fermentation equipment is legion, with many special claims for particular effectiveness. Excepting the case of filamentous growth, however, it is difficult to see how different agitator designs can affect the performance of the organisms so long as the basic requirement for complete mixing—uniformity of the reaction mass—is satisfied.

The work reported here was undertaken to provide experimental evidence for this view.

Experimental

Fermentation System

Aerobic propagation of baker's yeast was selected for the examination of agitation effects. All fermentations were carried out in the manner described earlier by Hixson and Gaden.[4] A medium of diluted Diamalt syrup (nondiastatic), supplemented with ammonium phosphate and adjusted to pH 4·5 with sulphuric acid, was used. The fermenter used was similar to others described in the literature,[2,4,6] except that it was simpler in construction (sterility was not required). It consisted of a Pyrex glass jar with a sheet stainless steel head, carrying baffles and an air line with a fritted stainless steel disc sparger, fitted closely over the top.

Agitation was provided by either a turbine impeller or a special, high-shear 'Dispersator' (Premier Mill Corp.). Both are shown

in Fig. 1. The turbine impeller is similar to the type used in most commercial and pilot-plant fermentation equipment. The 'Dispersator' is one of many available devices for promoting

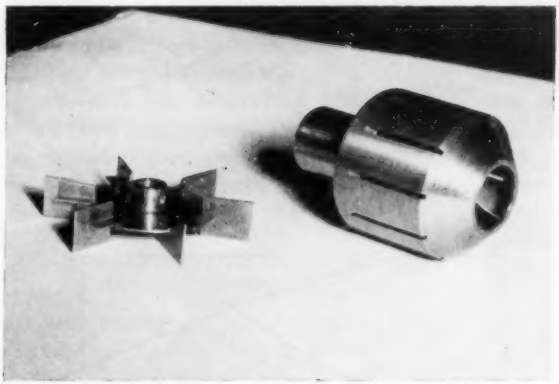

Fig. 1. Agitators: turbine (left) and shear types

fluid agitation together with high mechanical shear at right angles to the general flow pattern. It consists essentially of a hollow, slotted cylinder with an internal transverse flat blade. As it rotates the fluid is drawn in the bottom and discharged again through the slots into the fluid bulk.

In the 17 runs conducted, all fermentation conditions were maintained constant except for: (1) agitator speed varied from 0 to 1,500 rev/min, and (2) agitator type. Temperature was held at 30°C and the air rate as noted below. Ammonium hydroxide was added to keep the pH between 4·4 and 4·7. Sugar concentration, determined in the conventional manner,[7] and tissue weights, obtained by centrifugation, washing and drying to constant weight,[4] were periodically determined. Oxygen concentrations in the fermentation broth were determined amperometrically, again as previously reported.[4] The results of a typical run are shown in Fig. 2.

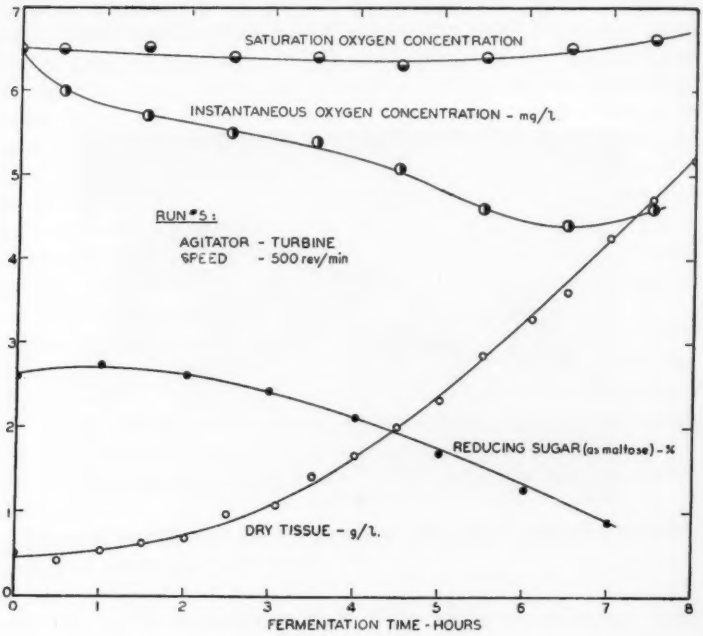

Fig. 2. Typical yeast propagation run

Elimination of Oxygen-transfer Effects

In order to evaluate agitation separate from aeration effects it is necessary to operate in such a manner that the effective oxygen concentration is independent of the degree of mechanical agitation. This can be done by maintaining oxygen concentration above the 'critical' level. It has been amply demonstrated[4,11] that the respiration rate, or oxygen demand, of yeast is not affected by oxygen concentration as long as the dissolved oxygen level is above a rather low, critical value. Several preliminary fermentations were conducted in which aeration was varied—without any mechanical agitation—until an air rate was found which would maintain the dissolved oxygen concentration at or above

25 per cent of saturation throughout the course of the run (8 h). The 25 per cent value was arbitrarily chosen to be considerably in excess of the critical which is somewhere around 5–10 per cent of saturation.[4] For the fermenter arrangement used (fritted stainless steel sparger) an air rate of 10 standard l./min, measured at 14·7 lb/in^2 gauge and 70°F, was found to be adequate.

Mechanical power inputs were measured with a dynamometer, the fermenters being batched with 10 l. of water and aerated at 10 standard l./min (see above) for the tests. A plot of power input, in conventional terms of h.p./100 gall, *versus* agitator speed is of the expected form with different exponents for the two agitator types (Fig. 3). There is, of course, some power contribution from the sparged gas. This could be calculated but the

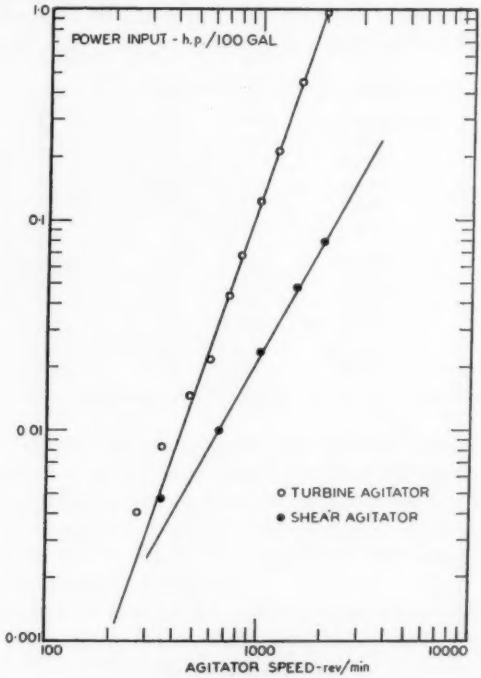

Fig. 3. Agitator power characteristics

use of a fritted sparger of approximate and varying pore size makes such a calculation very approximate at best. Since this power contribution is, in any event, extremely small when compared with that of the agitator,[1] it has been neglected here.

Results

Yeast growth, measured in terms of tissue weight, generally follows an exponential relationship during aerobic propagation.[10] The total weight of yeast (w) present at any time (t) is related to that present at the end of the preliminary 'lag' period (w_0) by the expression:

$$w = w_0 e^{kt}$$

The specific growth rate constant, k, should be dependent upon the availability of nutrients (including oxygen) and the physical

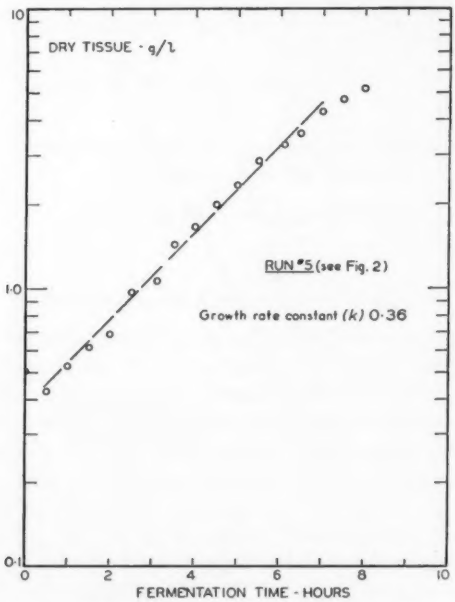

Fig. 4. Determination of growth rate constant

conditions of the propagation system. With temperature and pH fixed, and oxygen in abundant supply, changes in the growth rate constant with agitator power will reveal specific effects of agitation, if any, on the organism-medium interaction.

By plotting the logarithm of tissue weight (g dry tissue/l.) against time (Fig. 4), growth rate constants were determined for all runs (Table I). In determining k values, greatest weight is

Table I

	Agitation			Growth rate constant (k)
	Run	rev/RPM	h.p./100/gal	h^{-1}
No mechanical agitation	1	—	0	0·33
	2	—	0	0·29
Turbine type agitator	3	250	0·0019	0·33
	4	250	0·0019	0·34
	5	500	0·015	0·36
	6	500	0·015	0·37
	7	740	0·050	0·38
	10	915	0·093	0·39
	14	985	0·115	0·38
	13	1,215	0·220	0·44
	16	1,500	0·400	0·45
Shear type agitator	15	350	0·0034	0·36
	17	400	0·0044	0·37
	8	760	0·014	0·36
	9	1,065	0·026	0·39
	11	1,500	0·048	0·36
	12	1,500	0·048	0·39

given to the middle portions of the growth curve—say 2–6 h. The first hour or so of the propagation period comprises an adjustment or transition (lag) period while later in the fermentation substrate exhaustion may be encountered. This last factor is most clearly evidenced by an upturn in the level of dissolved oxygen in the medium (Fig. 2).

Growth rate constants for yeast propagation (average values where duplicate runs were made) are plotted against power input in Fig. 5. Three features of this relationship are worthy of note.

First, agitator type apparently has no influence on growth rate.

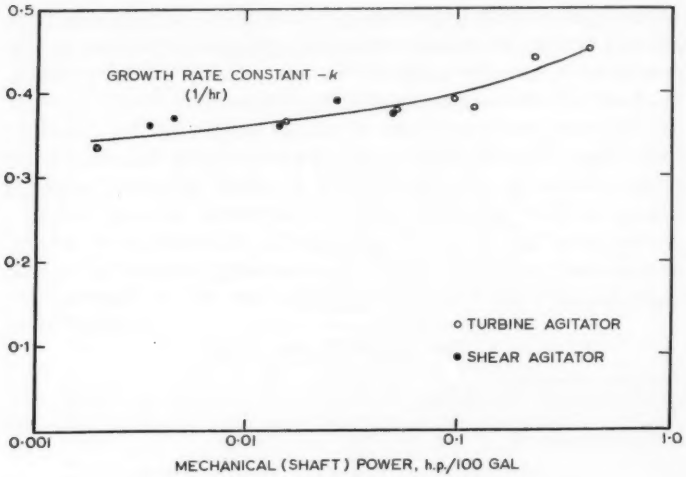

Fig. 5. Effect of agitation on growth rate

So long as correlation is based on power input—and not simply agitator speed—agitation effects in the system are insensitive to agitator design. In other words we need concern ourselves only with the degree of turbulence introduced in a fermenting mash and not with the manner of its introduction. (Note: The effectiveness of gas dispersion—not a factor here—is very much influenced by agitator design. Only agitation effects within the fluid menstrum itself are considered here.)

The second and third features of the relationship are best stated together. It is evident that mechanical power input does influence growth rate, but the observed change is not a very substantial one; growth rate constant increases only about 25 per cent for a 1,000-fold increase in power input.

If the basic premise of these experiments—that oxygen supply was never limiting—is legitimate, then the benefits of increased agitation must result from some direct influence on the cell-medium interaction. For a simple Newtonian fluid (i.e. free from complex mycelial networks) like this, only two effects are possible: (1) increased power ensures a more homogeneous, more perfectly

mixed, batch, and (2) the rates of transfer of materials between individual cells and their immediate fluid environment is enhanced.

While the experiments reported here cannot positively distinguish between these two factors, available evidence weighs heavily in favour of the first alternative. For example, Rhodes and Gaden[5] have shown that increased agitation (by shaking) has little effect on mass transfer to fine resin particles from a suspending liquid. Transfer of nutrients, etc., between fluid and suspended yeast cells should be even less affected by stirring of the bulk liquid because of the similarity in density between the two phases and the much smaller size of cells compared to the particles studied in reference 5.

It is likely, then, that the small improvement in growth rate

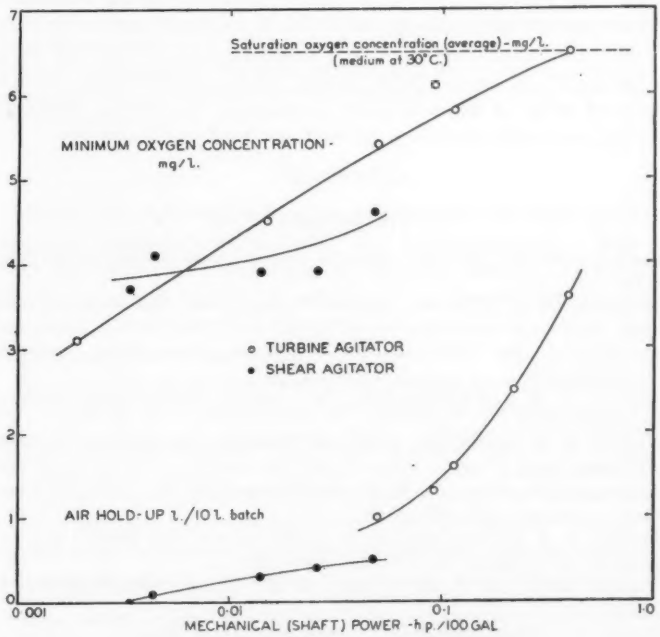

Fig. 6. Comparative aeration efficiencies

with increased agitation of the medium can be most readily attributed to improved mixing of the bulk liquid. 'Perfect mixing' is, indeed, an ideal, difficult of attainment in most agitation systems.

One final point of interest is the relative aeration (oxygen absorption) efficiences of the two different agitators. To illustrate this, air hold-up, in l. per 10 l. batch, measured at 8 h, and the minimum oxygen concentration reached in the fermentation are both plotted in Fig. 6. On the basis of these indices the turbine is clearly the better aerator with the disparity becoming greater as agitator speed is increased.

Conclusions

Increased agitation of fermentation medium, under conditions of abundant oxygen supply, improves the specific rate of growth of yeast, but only slightly. This improvement is believed to be due primarily to better 'bulk mixing' of the mash rather than to improved rates of mass transfer (nutrients, metabolic products, etc.) between cells and their immediate fluid environment.

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Problems in Large-scale Culture of *H. pertussis**

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Summary. A study of factors affecting the reproducibility and yields in cultivations of *H. pertussis* (the whooping cough organism). The search for suitable media led to the development of a new kind of blood medium suitable for pre-cultures. Sterility of the blood additive could be ensured by filtration, and contamination on starting the freeze-dried cells was avoided by the use of a special kind of ampoule.

In the course of studies of different cultivation techniques a 6,000 ml 'pulsaerator' was found to be ideal for the preparation of ordinary small batches. Inoculation density, aeration and other factors affecting the yield were studied and a culture cycle suitable for preparing 500 l. batches was developed.

Introduction

The problems involved in the preparation of large batches of pertussis bacteria are important for several reasons. Not only does the practical production of vaccines for the prevention of whooping cough call for a solution of these problems but basic research, particularly in the field of immunology, may require large quantities of material.

A case in point is the study, undertaken here in collaboration with a group of chemists,[1] of the biological activity of fractions from disintegrated pertussis bacteria. With the aid of column electrophoresis and ion exchange techniques it was possible to obtain a large number of fractions. Each of these should contain enough material for a complete set of tests concerning agglutination titres, toxicity, immunogenicity, pyrogenicity, and character in gel precipitation and immuno-electrophoresis. It is

* The research reported here has been made possible through the support and sponsorship of the U.S. Department of the Army, through its European Research Office.

obvious that substantial quantities of starting material were necessary to meet this requirement. Both continuous and batch cultivation techniques were considered. This paper presents an account of the problems encountered in setting up a batch process in the 500 l. range.

The basic questions were:

(1) What media should be used to permit good economy without involving undesirable changes in the phase of the strains, including antigenicity, toxicity, pathogenicity and virulence?

(2) How should good reproducibility and satisfactory sterility be secured?

(3) What cultivation techniques and subculture process would give high cell yields?

It is probable that there are many answers to the three questions but we feel that it might be helpful to others if we describe the technique which in our hands gave satisfactory results. This will be discussed under the three headings: basic media, sterility and cultivation technique, corresponding to the questions just formulated.

Technique

Basic Media

For a comprehensive discussion of the media previously used the reader is referred to the papers by Hornibrook,[2] Rowatt,[3] and Billaudelle.[4] As early as 1939 Hornibrook[2] used starch in media for *Haemophilus pertussis*, and both Proom[5] and Rowatt[3] later confirmed the importance of this additive. We also found that starch was necessary for satisfactory yields, both from the qualitative and quantitative points of view. As could be expected from Jobb's and Tomlinson's[6] experiments with different sugars, starch could not be substituted by simple mono- or oligosaccharides.

Solid and liquid media containing sheep and rabbit blood, as used by Billaudelle,[4] are particularly useful because they permit extended subcultivations without loss of virulence (by the intracerebral route) and antigenicity. Their use, however, poses a sterility problem, so they will be discussed later. As a rule, simpler liquid media are used for submerged cultures.

The first medium was a modification of that used by Cohen and Wheeler[7] and described by Arvidsson and Ullberg-Olson.[8] It gave excellent growth, but was based on special ingredients (casein hydrolysates, yeast dialysate) not readily available and also quite difficult to standardize. As good reproducibility was an absolute pre-requisite in our experiments, a medium based on Casamino acids (Difco) was tried. The following composition gave excellent growth:

Fluid medium for growth of *H. pertussis*. (Medium A):

Ingredients		per litre
Bacto-Casamino acids	(Difco)	10 g
Yeast extract	(Difco)	2.5 g
Soluble starch p. anal.	(Merck)	1.5 g
$MgCl_2.6H_2O$ 25%	(Merck)	1·6 ml
$CaCl_2$ 10%	(Merck)	0·1 ml
$FeSO_4.7H_2O$ 1%	(Merck)	1·0 ml
$CuSO_4.5H_2O$ 1%	(Merck)	0·05 ml
0·01% l-Arginine		1·0 ml
0·005% l-Histidine		
KH_2PO_4 (Sörensen)		0·5 g
pH = 7·8		

Medium B was the same as Medium A but with Bacto-Casamino acids Technical, instead of Bacto-Casamino acids.

Since Casamino acids are quite expensive the technical product was tested, and this medium (B) gave a highly satisfactory growth and was used in most experiments reported in this paper.

Sterility

To ensure good reproducibility it is not only important to guarantee uniformity in different batches of media, but also the inocula must have identical histories. This was achieved by resorting to a bank of ampoules containing freeze-dried bacteria originating from a single culture. To guard against immunological deterioration this culture had to be grown on a blood medium. It proved very difficult, however, to obtain consistently uncontaminated material even when the sterility of the medium was tested by overnight incubation at 37°C. A strict sterility technique aiming at eliminating contamination from the air did not improve matters, and the troubles were finally traced

back to two causes: unsterile batches of blood, and transfer after freeze-drying.

It is true that small amounts of rabbit and sheep blood can be obtained that are satisfactory with regard to sterility (cardiac and jugular puncture), but the problem becomes difficult when larger quantities are needed. A natural solution is then to haemolyse the blood and filter for sterility in the following manner:

8,000 ml of sheep blood from the carotid artery was obtained at ± 0°C from animals being slaughtered. 1 ml 5 per cent heparin was added per 1,500 ml blood. After storage for 15 h at $+4$°C, the blood was centrifuged for 2 h at $4{,}000 \times g$ in a water-cooled 13 l. 'International' serum centrifuge. The sediment was haemolysed by the addition of 2 volumes of *aq. dest.*, the process being speeded up by freezing once in a -60°C alcohol bath followed by thawing at $+10$°C. The stroma was removed by centrifugation for 1 h at $4{,}000 \times g$ and discarded. The haemolysate and the plasma fraction were then separately freeze-dried and stored at $+4$°C. About 810 g haemolysate and 470 g plasma were obtained. When needed for the preparation of media, quantities corresponding to those in whole blood (14 g haemolysate and 7·82 g plasma per 100 ml 'blood') were mixed and passed through a Seitz filter.

Additives were tested in different concentrations, both in liquid and solid media. Table I summarizes the principal media employed.

Table I

	Composition for approximate additive level of, %			
	25	12·5	10	6
Basic materials				
Cohen–Wheeler type medium (2% agar)	100·0	100·0	100·0	100·0 ml
Soluble starch (Merck) (7% agar)	4·2	4.2	4.2	4·2 ml
* *Additives*				
(1) Native blood, sheep	13·0	6·5	5·0	3·0 ml
rabbit	13·0	6·5	5·0	3·0 ml
(2) Reconstituted sheep blood	26·0	13·0	10·0	6·0 ml
(3) Plasma 7·82% in *aq. dest.*	26·0	13·0	10·0	6·0 ml
(4) Hb portion 14% in *aq. dest.*	26·0	13·0	10·0	6·0 ml

* (1–4) represent different alternatives.

Plasma or haemolysate alone (in 6, 10 and 12·5 per cent concentration) were not enough to give satisfactory growth. To ensure this, as well as stability in virulence and agglutinability through a number of passages, it proved necessary to increase the concentration of blood additive to 25 per cent. The following experiment (Table II) shows that this medium gives satisfactory results even if the agglutination titre does not reach the value (1/102,400) that can be obtained by a more complex technique involving considerable contamination risks.

Table II

Treatment	Amount of growth	Growth phase	Agglutination* titre	Virulence† (LD_m 10 g mouse)
Starting material	satisfactory	I	1/102,400	about 100 bact.
One passage on solid:				
Normal blood 10 or 25%.			1/102,400	
Reconstituted blood 25%.		I	1/25,600	
Four passages on fluid and solid alternatively:				
Normal blood 10 or 25%.		I	1/12,800	
Reconstituted blood 25%.		I	1/6,400	

* The agglutinability was tested with a hyperimmune serum towards strain 44 122/7 on the formol-antigens. None of these agglutinated in normal rabbit serum diluted 1/100.

† By the intracerebral route. It should be noted that the deterioration, as a consequence of the cultivation, expressed itself not only in the larger dose necessary to cause death within 14 days but also in a 'time drift' in the response curve. Actually a highly virulent and a deteriorated culture might give the same LD_m in 14 days, but in the first case the animals died between the fifth and ninth days, in the second between the eighth and twelfth days.

Cultivation Technique

As mentioned above the filtered haemolysate media were particularly useful in making it possible to prepare a definitely sterile batch of virulent, antigenically complete, freeze-dried cultures. Originally a batch of 250 ampoules was prepared from a seemingly sterile Cohen–Wheeler culture stemming from strain 44 122/7 growing on a normal solid blood medium in a Petri dish. In spite of the rigorous technique employed no less than 5–10

per cent of those ampoules were contaminated. A new batch of the same strain was therefore prepared from a C–W suspension of bacteria grown on a solid filtered haemolysate medium. For this culture a new kind of ampoule, reducing the contamination risk on opening, was also devised (Fig. 1).

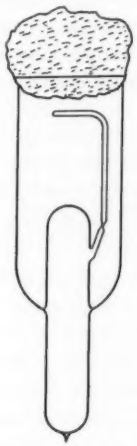

Fig. 1. Pre-culture tube for freeze-dried starting material

The ampoule is a double tube made up of a regular test tube with a smaller tube inserted through the bottom. To a weakened portion of the wall of the small tube a short glass rod is attached. Its free end is close to the stopper of the large tube.

After sterilization of the stoppered ampoule the culture is placed in the small inner tube. When freeze-drying is concluded this tube is vacuum sealed by melting. When the time has come to use the material to start a culture cycle, 5–10 ml of liquid medium is placed in the outer test-tube. After incubation at 37°C for 24 h to check sterility, this medium is inoculated with the dry material by simply breaking the ampoule with the aid of the glass rod, which is moved by the stopper.

Transfers from primary cultures grown at 37°C for 48 h to larger culture volumes were done either by pipetting, employing ordinary precautions, or with the aid of sterile syringes when the cultivation equipment was fitted with rubber membranes for perforation. This technique and the methods for handling large culture volumes are described in a paper by Hedén[9].

Several different methods for carrying out the secondary culture were tried. First, a set of rotary shaker experiments was carried out both in simple liquid medium and in Tyrrell, Russel and Gerhardt's[10] biphasic liquid/solid system. Cultivation at 37°C for 48 h in a 250 ml Erlenmeyer flask containing 50 ml solid blood medium and 10 ml Cohen–Wheeler liquid medium did not, however, yield particularly satisfactory results.

Ordinary batch cultivation techniques were also studied, and a large number of cultivations were carried out in pulsaerators.[12, 11] This type of unit was chosen for three reasons: (1) it permitted good aeration in the late phases of growth when cultures foamed badly; (2) its single filter (for incoming and outgoing air) was highly efficient; (3) it permitted some changes in 'active aeration' without markedly affecting the bubble-stirring. This latter characteristic was particularly important in this case, for one could suspect that CO_2 stripping might have undesirable effects. The possibility of such a factor was indicated by the differences in yield in units of varying design.

In spite of a well standardized routine it was at first very difficult to reproduce yields in different experiments. This variability was investigated and such factors as light and the toxic products in the air were ruled out. An important factor was then found to be the size of the inoculum. This is illustrated by the data of Table III, which summarizes a series of experiments carried out with 1·5 l. pulsaerator units. They were inoculated with different quantities (1·5–12 ml) of a sheep blood culture and the stationary phase yield was determined after 72 h at 37°C:

Table III

Initial density: organisms/ml	$0{\cdot}16 \times 10^9$	$0{\cdot}32 \times 10^9$	$0{\cdot}64 \times 10^9$	$1{\cdot}28 \times 10^9$
Yield: organisms/ml	5×10^9	10×10^9	12×10^9	16×10^9

The yield in a 6,000 ml unit of the type illustrated in Fig. 2, using the lowest inoculation density shown above, was no less than 36×10^9/ml, and it soon proved that the bigger units constantly gave better yields ($30–40 \times 10^9$/ml) in 48–72 h than the smaller pulsaerators ($10–15 \times 10^9$/ml). Both stirring and aeration, as measured by the sulphite oxidation technique, are better in the latter type, but a simple inverse relationship with the yield obviously does not exist. If, for example, the aeration/stirring

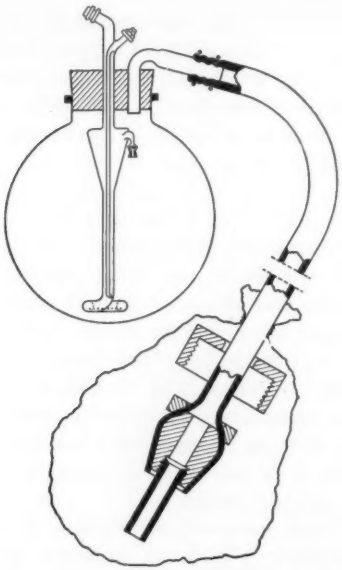

Fig. 2. Pulsaerator flask for 5–6 l. *H. pertussis* culture. The paper-wrapped stericonnector element, which is used for attaching flask to 1,000 l. fermenter, is drawn to a larger scale than that of the flask

is increased by a reduction in culture volume (in the 1,500 ml units) the yield actually went up. This is illustrated by the following experiment (Table IV), where all units were inoculated with the same culture to an initial density of 2×10^8/ml:

Table IV

Type of unit	Culture volume, ml	72 h density (bacteria/ml)	Phase
1,500 ml type	2,000	9×10^9	IV
	1,500	12×10^9	I (mostly)
	1,000	12×10^9	I
	500	15×10^9	I
6,000 ml type	6,000	30×10^9	I
	6,000	28×10^9	I
	6,000	36×10^9	I
	6,000	26×10^9	I

No simple explanation has been found for the phenomenon just mentioned. Some preliminary experiments with 5 ml pulsaerators where the residual volume has been varied indicate that stripping may be a factor of importance. The metabolites in question need not, however, include CO_2, which does not markedly improve growth when added to the gas phase. In this connection it should perhaps be mentioned that Ungar *et al.*,[13] have found that bubbling air through the medium preferentially selects the avirulent part of the population. No drop in virulence was, however, observed in our 6,000 ml pulsaerators, material from which also gave a satisfactory mouse protection test. At the same time, the special character of this test, particularly the intracerebral injection, should be kept in mind (*cf.* Standfast[14]).

It was found that the 6,000 ml units could be inoculated directly with a 10 ml haemoglobin plasma, 48 h culture, started from a freeze-dried culture. This simple technique yields a rigorous phase I culture, which is well suited for starting pilot-plant cultivations.

A conventional starting technique (freeze-dried culture→liquid blood medium, 24–48 h→solid blood medium, 24 h→liquid Cohen–Wheeler medium, small scale, 48 h→6,000 ml Cohen–Wheeler liquid medium, 48 h) takes longer (168–192 h) and involves bigger risks of contamination than the new technique, which is not only quicker (72–120 h) but also uses blood throughout. Preliminary experiments indicate that this can be of human as well as of sheep origin.

For stepping up the culture volume from the 6,000 ml stage, a round, 100 l. pulsaerator flask was first tried. Owing to some inexplicable difficulties with sterility, the use of this unit was, however, soon discontinued and cultivation was carried on instead in one of the 1,000 l., stainless steel fermenters in the pilot plant. If 500 l. of medium in this tank was inoculated with only one 6,000 ml culture of 26×10^9 bact./ml, a yield of about 7×10^9 bact./ml was obtained after 72 h at 37°C. The material was pure and the bacteria of the phase I type mixed with intermediary phase types. The agglutination titre was about 1:12,800 but the virulence was extremely low (> 10·000 bact./10 g mouse).

The importance of the size of the inoculum was also evident in the 500 l. scale. If the tank was inoculated with five—instead of one—6 l. batches, the yield actually reached 30×10^9/ml

(=about 800 g of bacterial paste). This material had the virulence of the original strain (=100 bact./10 g mouse) and gave the agglutination titre 1:12,800 in our standard serum.

The fermenter was fitted with a bottom stirrer consisting of a disc of 250 mm diameter fitted with 6 vertical blades, 50 by 50 mm in size. It was operated at about 300 rev/min and caused the formation of a vortex. This controlled foaming,[15] and hence no chemical foambreaker had to be added. The air over the vortex was changed at a rate of about 50 l./min.

Mass Cultivation Schedule

The techniques briefly outlined in the previous section all served as building blocks for establishing a suitable routine for large-scale cultivation of *H. pertussis*. This routine is best described in the form of a condensed schedule for a specific experiment.

Schedule

0 h: 5 ml medium is inoculated with a freeze-dried culture as described under Cultivation technique.

24 h: Gram-staining, subculture on 25 per cent haemoglobin-plasma solid medium and possibly virulence test as a control.
After inspection of Gram specimens, inoculation of six 6,000 ml batches of A or B medium in pulsaerators.

72 h: Gram-staining, agglutination test, inoculation of 500,000 ml unit.

120 h: Harvesting.

Discussion

The experiments described were necessary as steps in making available large quantities of *H.pertussis* material, satisfactory from the point of view of virulence and antigenicity. The aim was physicochemical fractionation, but the same need for large quantities of material has long been felt by those who work with vaccine production.[16] The Bordet–Gengou solid medium first tried had two drawbacks: great susceptibility to contamination

when handled in bulk, and blood components that were allergenic to man and difficult to exclude from the bacterial suspensions.

Considering the greater ease with which liquid media can be handled, this type of culture was then tried. Hornibrook,[2] Cohen and Wheeler[7] and others[8] also obtained good results with liquid media on a casein-hydrolysate base. It proved impossible, however, to preserve the virulence, antigenicity and agglutinability of the delicate phase I bacteria in those media for any length of time. To satisfy the requirements just mentioned a rather complicated system of subcultures and controls had to be employed—a system well known to everybody working with *H. pertussis*.

Freeze-drying is an important first step in the stabilization of the process. This may also include a mouse-brain passage to preserve or regain virulence. Since this step introduces a sterility hazard, an improvement was made by replacing the animal passage with a series of liquid and solid sheep and sheep/rabbit blood media. Fresh animal blood in the media might also cause occasional contamination and elimination of the blood components thus seemed to be necessary. At the same time virulence, pathogenicity, antigenicity, etc., could not be lost. We found that a haemolysate medium could be used to advantage in this connection, and the different cultivation steps were investigated from the point of view of sterility and yield of satisfactory material. The simple subculture chain which follows was finally elected: Freeze-dried culture→sterile haemolysate media →5–6 l. culture→500 l. culture in Cohen–Wheeler type medium.

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The Theory of Scaling up Laboratory Data for the Sedimentation Type Centrifuge

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Summary. The mathematical theory of the sedimentation type of centrifuge is developed in terms of unhindered settling of a single particle that has reached equilibrium velocity. From this is derived the sigma value for several of the different forms of sedimentation type centrifuge which is the calculated equivalent area of a settling tank theoretically capable of doing the same amount of work in a unit gravitational field. The limitations controlling the accuracy of scale up between centrifuges of similar geometry and between centrifuges of substantially different geometry are discussed.

Introduction

Apparatus using centrifugal force for resolving multi-component systems can be divided into two major categories, and a third which may be considered a combination of the other two:

(1) solid wall centrifuges in which the separation is by subsidence,
(2) perforate wall centrifuges in which the solid phase is supported on a permeable surface through which the liquid phase is free to pass, and
(3) combinations of the two in which the primary concentration is effected by sedimentation followed by subsequent drainage of the liquid phase away from the solid phase.

Each of these has its gravitational analogue: (1) the settling tank or thickener; (2) the vibrating or rotary screen; and (3) the rake classifier.

In the case of the solid wall centrifuge, as is true of the settling tank, there are two types of forces opposing the movement of the particles of the discontinuous phase through the continuous fluid

phase. For small particles, moving not too rapidly, the viscous resisting force is directly proportional to the velocity of sedimentation. For larger particles moving rapidly, when their Reynolds number, $dv\rho/\eta$, is greater than the order of unity, the viscous resistance becomes negligible compared to the turbulent resistance which is proportional to the square of the velocity of sedimentation.

General Analysis

In analysing centrifuge performance, when a high degree of clarification or separation is important, the behaviour of the smallest particles in the system is usually the controlling factor. We may, therefore, consider viscous resistance to be of prime importance although a knowledge of the factors involved in turbulent resistance is also of interest and will be given secondary consideration.

The effective force acting on a given particle in a centrifugal field is

$$F_1 = (m - m_1)\omega^2 r \tag{1}$$

where m = the mass of the particle, m_1 = the mass of fluid it displaces, ω = the angular velocity about the axis of rotation, and r = the distance of the particle from the axis of rotation.

If the particle is a sphere, the force is

$$F_1 = \frac{\pi}{6} d^3 \Delta\rho \ \omega^2 r \tag{2}$$

where d = the diameter of the particle and $\Delta\rho = \rho - \rho_1$ = the difference between the density of the particle and that of the fluid in which it is suspended.

The force opposing the sedimentation of the particle, assuming that it is small and does not move too rapidly, is given by Stokes' law

$$F = 3\pi\eta d v_s \tag{3}$$

where η = the viscosity of the fluid phase, and v_s = the velocity of the particle moving through it.

When the force causing sedimentation reaches equilibrium with the resisting force, i.e. when v becomes constant,

$$v_s = \frac{\Delta\rho d^2 \omega^2 r}{18\eta} \tag{4}$$

When the particle is in the gravitational field

$$v_g = \frac{\Delta\rho d^2 g}{18\eta} \tag{5}$$

The simplest form of a continuous centrifuge, *cf.* Fig. 2, is a rotating cylinder with end caps, provided with means for being fed fluid at one end and discharging it from the other. In such a device v_s is the velocity with which the particle approaches the bowl wall (or if $\Delta\rho$ is negative, the liquid surface). If the thickness, s, of the liquid layer is small compared to the radius of the cylinder, then v_s will be approximately constant and the distance a given particle will settle during the time the liquid in which it is suspended is in the bowl

$$x = v_s t = \frac{\Delta\rho d^2 \omega^2 r}{18\eta} \cdot \frac{V}{Q} \tag{6}$$

where t = time,

Q = rate of flow of liquid through the bowl,

V = volume of liquid in the bowl = $\pi l\,(r_2{}^2 - r_1{}^2)$. (6a)

If x is greater than the initial distance of the given particle from the wall of such a rotor, it will be deposited against the wall and removed from the liquid phase. In an ideal system when $x = s/2$, half the particles of diameter d will be removed from suspension and half will not. This condition will be considered the cut-off point and at cut-off, by substituting $x = s/2$ and rearranging equation (6).

$$Q = \frac{\Delta\rho d^2}{9\eta} \cdot \frac{v\omega^2 r}{s} \tag{7}$$

from which the critical diameter

$$d = \left(\frac{9\eta Q}{\Delta\rho} \cdot \frac{s}{V\omega^2 r}\right)^{\frac{1}{2}} \tag{8}$$

for particles, half of which will be removed, can be calculated.

Referring back to equation (7), it will be noted that the factors in the first group of the right-hand side of the equation are concerned only with the parameters of the system being processed; density, diameter and viscosity. The factors in the second group are concerned only with the parameters of the centrifuge, volume, rotational speed, radius and settling distance.

Equation (7) may be rewritten

$$Q = 2v_g\Sigma \tag{9}$$

in which

$$v_g = \frac{\Delta\rho d^2 g}{18\eta} \tag{5}$$

and

$$\Sigma = \frac{V\omega^2 r_e}{g s_e} \tag{10}$$

in which r_e and s_e are the effective radius and settling distance of any centrifuge of this type, and Σ is an index of centrifuge size. It is, in fact, the calculated equivalent area of a settling tank theoretically capable of doing the same amount of work in a unit gravitational field.

Theory for Different Types of Centrifuges

Let us consider different types of centrifuges operating on a system in which viscous resistance to particle motion is the controlling factor.

(1) The laboratory 'bottle' or batch centrifuge in which cylindrical containers are rotated with the axis of the cylinder at right angles to the axis of rotation.

(2) The continuous flow tubular type in which the settling distance is not small with respect to the radius.

(3) The disc type in which the settling distance is small with respect to the radius and in which the discs are sloped to permit radial movement of the sedimented solids across them.

The bottle centrifuge, Fig. 1. For this, equations (4) and (5) may be re-arranged,

$$v_s = v_g \frac{\omega^2 r}{g} = \frac{\mathrm{d}r}{\mathrm{d}t} \tag{11}$$

Then, if r = distance of the particle from the axis of rotation, r_1 = radius of liquid surface in bottle, r_2 = radius at surface of sedimented cake, and t = total centrifuging time, we can re-arrange equation (11) and integrate:

$$\int_r^{r_2} \frac{\mathrm{d}r}{r} = \int_0^t \frac{v_g \omega^2}{g} \mathrm{d}t \tag{12}$$

from which

$$\ln \frac{r_2}{r} = \frac{v_g \omega^2 t}{g} \tag{13}$$

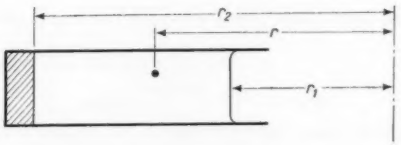

Fig. 1. Particle in bottle centrifuge

At the 50 per cent cut-off point where half of the particles would be sedimented assuming them all to be the same size

$$A\,(r-r_1) = A\,(r_2-r) \tag{14}$$

A = the cross-sectional area of the bottle and

$$\therefore \quad r = \frac{r_2+r_1}{2} \tag{15}$$

Also,

$$t = \frac{V}{Q} \tag{16}$$

Q = Effective 'flow rate' through the bottle (ml per min). Substituting equations (15) and (16) into (13) and re-arranging,

$$Q = 2v_g \frac{\omega^2 V}{2g \ln \left(\dfrac{2r_2}{r_1+r_2}\right)} \tag{17}$$

from which

$$\Sigma = \frac{Q}{2v_g} = \frac{\omega^2 V}{4{\cdot}6g \log \left(\dfrac{2r_2}{r_1+r_2}\right)} \tag{18}$$

or more conveniently

$$\frac{Q}{\Sigma} = \frac{4{\cdot}6g \log \left(\dfrac{2r_2}{r_1+r_2}\right)}{\omega^2 t} \tag{19}$$

The time required to sediment all particles of diameter d can be determined by integrating dr/r in equation (12) between the limits r_1 and r_2. This may be regarded as the special case when $r = r_1$, and

$$\ln r_2/r_1 = \frac{v_g \omega^2 t}{g} \tag{20}$$

For spherical particles, from equation (5) and re-arranging

$$t = \frac{18\eta \ln r_2/r_1}{\Delta \rho d^2 \omega^2} \tag{21}$$

The continuous flow tubular type. A typical tubular bowl is shown in Fig. 2. A particle at radius r and distance z from the bottom (inlet end) has two motions:

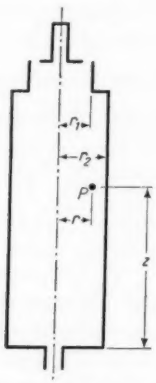

Fig. 2. Particle in tubular bowl centrifuge

(1) A radial velocity due to centrifugal force (equation 11)

$$\frac{dr}{dt} = \frac{v_g \omega^2 r}{g} \tag{22}$$

(2) A linear velocity due to the flow of liquid through the bowl, which is assumed to be uniform across its cross-section,

$$\frac{dz}{dt} = \frac{Q}{\pi(r_2^2 - r_1^2)} = \frac{Ql}{V} \tag{23}$$

from which

$$\frac{\mathrm{d}r}{\mathrm{d}z} = \frac{V}{Ql} \cdot \frac{v_g \omega^2 r}{g} \tag{24}$$

Integration of this equation, within the limits $r_1 = r$, at $z = 0$, and $r = r_2$ at $z = l$, gives

$$\ln\left(\frac{r_2}{r}\right) = \frac{V v_g \omega^2}{Qg} \tag{25}$$

The 50 per cent cut-off corresponds to

$$\pi(r_2^2 - r^2) = \pi(r^2 - r_1^2) \tag{26}$$

and

$$r = \left(\frac{r_2^2 + r_1^2}{2}\right)^{\frac{1}{2}} \tag{27}$$

By re-arrangement, and using equation (27), equation (25) becomes

$$Q = 2\, v_g \cdot \frac{V\omega^2}{g} \cdot \frac{1}{\ln\left(\dfrac{2r_2^2}{r_2^2 + r_1^2}\right)} \tag{28}$$

for which the approximation, within a maximum error of 4 per cent, is

$$Q \simeq 2v_g \frac{V\omega^2}{g} \left(\frac{\frac{3}{2}r_2^2 + \frac{1}{2}r_1^2}{r_2^2 - r_1^2}\right) \tag{29}$$

and by comparison with equation (10)

$$\frac{r_e}{s_e} = \frac{(\frac{3}{2}r_2^2 + \frac{1}{2}r_1^2)}{(r_2^2 - r_1^2)} \tag{30}$$

in which the effective values are

$$r_e = \frac{3r_2^2 + r_1^2}{4r_2} \tag{31}$$

and

$$s_e = \frac{r_2^2 - r_1^2}{2r_2} \tag{32}$$

From equations (29), (9) and (6a),

$$\Sigma = \frac{2\pi l \omega^2}{g} (\tfrac{3}{4}r_2^2 + \tfrac{1}{4}r_1^2) \tag{33}$$

In a similar manner, the Σ value of the tubular bowl in a system where turbulent resistance to motion is the controlling factor can be derived from the basic equation of resistance to turbulent motion

$$F = K\frac{\pi}{4}\rho_1 d^2 v_g^2 \tag{34}$$

K = a constant having a value of approximately 0·5 and dimensions of $1/g$

from which

$$v_s = \left(\frac{2}{3}d\cdot\frac{\Delta\rho}{Kg\rho_1}\cdot\omega^2 r\right)^{\frac{1}{2}} \tag{35}$$

$$Q = 2\left(\frac{2}{3}\frac{d\Delta\rho}{K\rho_1}\right)^{\frac{1}{2}}\cdot\frac{V}{s}\left(\frac{\omega^2 r}{g}\right)^{\frac{1}{2}} \tag{36}$$

$$v_g = \left(\frac{2}{3}d\cdot\frac{\Delta\rho}{K\rho_1}\right)^{\frac{1}{2}} \tag{37}$$

$$\Sigma = \frac{V}{s_e}\left(\frac{\omega^2 r_e}{g}\right)^{\frac{1}{2}} \tag{38}$$

and

$$\Sigma = 2\pi l\omega\left(\frac{r_2(\frac{3}{4}r_2^2+\frac{1}{4}r_1^2)}{g}\right)^{\frac{1}{2}} \tag{39}$$

It will be observed from a comparison of equation (39) with equation (33) that while the same elements are involved in the calculation of Σ, the exponents are quite different. On the other hand, in comparing equations (5) and (37) for the elements of v_g it will be observed that not only are the exponents different but g has been replaced by the reciprocal of the constant K, and η, the viscosity, by ρ_1, the density of the fluid phase.

The disc type. Sedimentation in a disc-type rotor can be treated theoretically by analysing the space between two adjacent discs on the assumption that the flow in such a rotor is uniformly distributed in all such spaces. Such a system is shown in Fig. 3. The discs extend from r_1 to r_2 and a particle is shown at position P. Its distance from the lower disc is y, and its distance from the outer edge of the disc, measured along the surface of the disc, is x. The

discs of a commercial centrifuge usually have horizontal flanges at their inner and outer diameters. These are present entirely for mechanical reasons, to improve the strength of the discs.

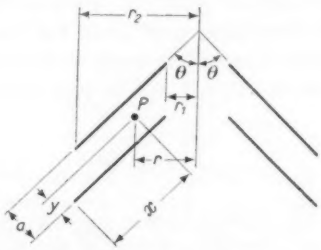

Fig. 3. Particle in disc bowl centrifuge.

They contribute nothing to clarification efficiency and are not considered in these measurements. The particle P has two motions,

(1) centrifugal force moves it away from the lower disc

$$\frac{dy}{dt} = \frac{v_g \omega^2 r}{g} \cos\theta = \frac{v_g \omega^2}{g} (r_2 - x \sin\theta) \cos\theta \qquad (40)$$

θ = the disc half angle, and

(2) it is swept along by the flow of liquid from r_2 towards r_1.

If there are n spaces between the discs in a given rotor and the total throughput is Q, then the flow between each pair of discs is Q/n and the average velocity across the distance a, between and normal to adjacent discs, at radius r is $Q/2\pi nar$. The velocity will be zero at the disc surfaces and reach a maximum somewhere between them. Let $f(y)$ be the ratio of liquid velocity at position y to the mean velocity. Then

$$\frac{dx}{dt} = \frac{Q}{2\pi nar} f(y) = \frac{Q f(y)}{2\pi na (r_2 - x \sin\theta)} \qquad (41)$$

and, from equations (40) and (41)

$$\frac{dy}{dx} = \frac{2\pi nav_g\omega^2}{Qg} \cdot \frac{(r_2 - x \sin\theta)^2 \cos\theta}{f(y)} \qquad (42)$$

Integration gives

$$\int_{y_2}^{y_1} f(y)\,dy = \frac{2\pi n a v_g \omega^2}{3Qg}(r_2^3 - r_1^3)\cot\theta \tag{43}$$

in which y_2 is the value of y at $x = 0$ where the particle enters the space between two discs and y_1 is the value of y at $x = (r_2 - r_1)$ cosec θ where the particle would leave the disc stack if it were not sedimented out and it will be just sedimented if $y_1 = a$.

Now

$$\int_{y_2}^{a} f(y)\,dy \tag{44}$$

is the fraction of the flow between $y_2 = y$ and $y_1 = a$ and all particles of diameter d in this portion of the flow are sedimented against the upper disc surface. Half the particles will be sedimented and half will not when the fraction is one half, and at cut-off,

$$Q = \frac{4\pi n v_g \omega^2}{3gC}(r_2^3 - r_1^3)\cot\theta \tag{45}$$

in which C = the average value for the integral of $f(y)$, and

$$\Sigma = \frac{Q}{2v_g} = \frac{2\pi n \omega^2 (r_2^3 - r_1^3)}{3gC\tan\theta} \tag{46}$$

The value and significance of C in equation (46) is not completely understood. If we assume the flow between adjacent discs to be symmetrical and laminar, then C equals unity only when either all particles of diameter d are sedimented or at cut-off when half the particles of diameter d are sedimented, i.e. when the value of y at $x = 0$ is either zero or $a/2$. However, the value of C is significantly different from unity when the particle starts from any other position and may be greater than unity even starting from the $a/2$ position if the flow pattern is disturbed by surface imperfections or by the accumulation of solids on the disc surfaces. This may also be true if the symmetry of the flow pattern is disturbed by the vector effect of centrifugal force or a change from a radial direction of flow due to conservation of angular momentum. The significance of this, and possibly other effects, has been confirmed by experimental data using satin clay in aqueous dispersion as the test medium.

Comparison of Centrifuges

Referring to equation (9) it will be observed that for a given test medium whose v_g is a constant, $Q/\Sigma = 2v_g$ = constant, and therefore it should be possible to compare various centrifuges with gravity settling systems on the basis that

$$\frac{Q_1}{\Sigma_1} = \frac{Q_2}{\Sigma_2} = \frac{Q_3}{\Sigma_3} = \text{etc.} \tag{47}$$

On tubular bowls over a fairly wide range of proportions of r_2, r_1 and l the determined Σ values were between 97 and 98 per cent of the calculated values. On disc bowls, when the flow was introduced at the outside diameter of the disc stack, the observed value was 55 per cent of theoretical. Both bowls were studied over the range of 1 to 20 per cent of unsedimented particles. The above is not to be construed as a criticism of the disc type of centrifuge. On many commercial applications its 'efficiency' does more nearly approach 100 per cent and conversely that of the tubular bowl may fall below the above mentioned 97 to 98 per cent value. The example selected does show that the relative actual performance characteristics of these two types of centrifuges may vary over comparatively wide limits, in a ratio approaching two to one, from their calculated characteristics.

The significant point is that data, in terms of Q/Σ, should not generally be extrapolated from the performance of one type of centrifuge to that of another.

In this connection, the effect of two other phenomena should be considered carefully.

The mathematical analysis has concerned itself with the sedimentation of a single particle in a field in which it is not affected by the pressure of other particles. For small particles, exhibiting Brownian movement, the diffusion constant

$$D = \frac{RT}{3\pi\eta N d} \tag{48}$$

where N = Avagadro's number, R = the gas constant and d = the particle diameter, may be appreciable and the applied centrifugal force must be great enough to overcome the 'threshold effect' before the laws of sedimentation will apply.

The second significant point to be gained from this is that, when dealing with very small particles, the extrapolation should not be made over too wide a range of centrifugal force even when dealing with centrifuges of similar proportions.

Referring back to equation (9) it will be observed that all succeeding calculations are based on the v_g of a given system being constant. While this is frequently true, the particles in many systems exhibit a degree of agglomeration with the passage of time and these agglomerates exhibit varying degrees of stability. A typical example is the precipitate of ferric hydrate, the stability of which is influenced to a great degree by the electrolyte concentration of the fluid phase. In a quiescent field such a precipitate shows a high value of v_g. When introduced into a rotating centrifuge bowl the abrupt change in angular velocity substantially changes the diameter of the individual particles. If the retention time in the centrifuge is short, even though the agglomerates do reform, in time, there is an apparent shift of Q/Σ to a smaller value for the same degree of clarification as the rotational speed of the centrifuge, and its shearing effect on the feed, increases.

A third significant point, which may be considered a combination, and in support of the first two, is that the extrapolation in many cases should only be made between centrifuges of the same type operating at approximately the same rotational speed.

Finally, it can be demonstrated that the plot of Q/Σ *versus* the proportion of unsedimented solids is theoretically a straight line when plotted in that order on logarithmic-probability paper when the particle size of the solids follows a normal Gaussian curve. No extrapolation should be undertaken in the range where such a plot deviates from linearity since this is a positive indication that the Σ value of the test centrifuge is deviating from theoretical.

Liquid-Liquid Systems

Up to this point, centrifugal performance has been discussed only in terms of the sedimentation of particles in a centrifugal field. Commercial centrifuges are also commonly used for the separation of immiscible liquids, such as oil and water. In such systems one phase is usually dispersed in the other and effective separation is considered to have been obtained when the required

proportion of the smaller dispersed particles has been separated out. Such a separation follows the same theory outlined above. In order to operate on such a system continuously it is necessary to keep the separated phases in a condition of hydrostatic balance. An arrangement for accomplishing this is shown in Fig. 4. It will be noted that this system is in apparent balance when the dimension $e-l$ times the density of the light phase equals the dimension $e-h$ times the density of the heavy phase. However, this only applies because the variation in the earth's gravitational field between points l and h is negligible. The general formula

$$\int_{r=e}^{r=l} \rho_l f(r)\, dr = \int_{r=e}^{r=h} \rho_h f(r)\, dr \tag{49}$$

is applicable. In the earth's gravitational field the values of $f(r)$ on each side of the equation are almost identical and cancel out so that

$$\rho_l(e-l) \simeq \rho_h(e-h) \tag{50}$$

In the centrifuge as illustrated in Fig. 4, this is not the case and

$$\frac{\omega^2\rho_l(e^2-l^2)}{2} = \frac{\omega^2\rho_h(e^2-h^2)}{2}, \tag{51}$$

or

$$E = \frac{\rho_h}{\rho_l} = \frac{e^2-l^2}{e^2-h^2} \tag{52}$$

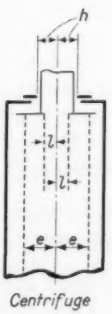

Fig. 4 (a). Separation in tubular bowl centrifuge

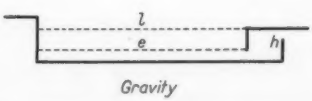

Fig. 4 (b). Separation in gravitational field

This general formula is equally applicable to the gravitational field if the dimensions e, h and l are measured from the centre of the earth, that is the point where $r = 0$, which is also the reference point from which these dimensions are measured for the centrifuge.

In continuous centrifuge rotors of both the tubular and disc types, provision is usually made to control the position of the interface (e line) by varying either the l or the h dimension of the bowl. The more common method is to vary the h dimension by the use of different sizes of ring dams or different lengths of heavy phase discharge nozzles. The r_1 of the light phase and the r_2 of the heavy phase become fixed dimensions of the rotor, while the r_2 of the light phase and the r_1 of the heavy phase, which generally correspond to the e line, can be varied to meet process requirements.

A given bowl running at a given speed therefore has a fixed total Σ value which is the sum of the variable Σ values of the portions of the bowl occupied by the light and heavy phases, Σ_l and Σ_h respectively.

For optimum use of a given separator bowl the e line must be controlled in accordance with the following relationships:

(1) Q_l/Σ_l is selected to give the required degree of purification of the light phase.

(2) Q_h/Σ_h is selected to give the required degree of purification of the heavy phase.

(3) Q (the total flow) $= Q_l + Q_h$

(4) Σ (the entire centrifuge) $= \Sigma_l + \Sigma_h$

When considering the operation of the tubular bowl as a separator on a given system (*cf.* Fig. 4) the optimum position of the e line can most easily be established by the trial and error method since variation of the e line within the limits l to e only affects Σ_l and Σ_h within the previously defined limits.

Liquid-Liquid Systems in Disc Bowls

When considering the disc bowl as a separator, another complication arises. The feed to such a bowl enters and is distributed through holes in the disc stack, Fig. 5. For optimum performance the e line must coincide with the location of these holes. If it

does not, then at least some of the Σ value of the fraction of the disc stack lying between the e line and the feed holes is not available for purification of the light (if the e line is outside of the holes), or of the heavy phase (if the e line is inside of the holes).

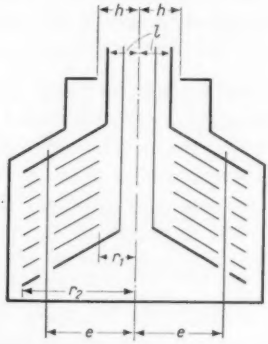

Fig. 5. Diagram of disc bowl separator

On a disc type separator bowl, therefore, not only must the proper ring dam adjustment be established, but the location of the feed distribution holes in the disc stack must coincide with the resulting e dimension.

Furthermore, where separation on a disc type rotor is being studied, either the Q/Σ values of both phases must be separately determined, or the extrapolation should only be made between centrifuges having similar Σ_l/Σ_h ratios.

Extrapolating Test Data

An examination of equation (46) indicates that the performance of a disc type centrifuge may be improved:

(1) by reducing the disc angle;

(2) by increasing the number of spaces between discs, n, which can be most easily done in a given bowl by reducing the space between adjacent discs, dimension a of Fig. 3.

In either case, the dimensions and nature of the solid particles present may become of critical importance. The disc angle must

be large enough to permit the sedimented solids to slide off them freely, and the spacing between discs must be large enough so that these accumulated solids as they slide along the disc surface do not interfere with the flow of liquid between discs.

To summarize, for simple tubular and disc clarifier and separator centrifuges, the following points should be considered before extrapolating test data from one size or type of centrifuge to another.

(1) Extrapolation should only be made in the area where the plot of Q/Σ *versus* proportion of unremoved impurity on a logarithmic-probability chart lies in a straight line.

(2) Extrapolation should not be made between different types of centrifuges (such as disc and tubular) unless the correction factors for the calculated Σ values of the particular centrifuges and the effect of the particular system on these correction factors are known.

(3) Extrapolation should not be made between centrifuges operating at different speeds unless it is known that the effects of diffusion and de-agglomeration will be negligible on the particular system.

(4) Extrapolation should only be made between disc centrifuges having a close similarity with respect to disc angle, disc spacing and, in separation problems, feed hole position.

Disc Centrifuges with Discharge of Sediment

Disc type centrifuges, because of their relatively small height to diameter ratio, are particularly suited for modification to permit the continuous or intermittent discharge of the sedimented solids through ports located around the periphery of the bowl. The inside of the bowl shell can be tapered or shaped to direct the solids to these peripheral ports without increasing the bowl diameter by an excessive amount, Fig. 6.

Two types of ports are generally used:

(1) Open nozzles through which there is a continuous flow of the heavy liquid carrying the sedimented solids with it.

(2) Valved ports which may be opened intermittently to permit the discharge of the accumulated solids with minimum discharge of the heavy phase liquid.

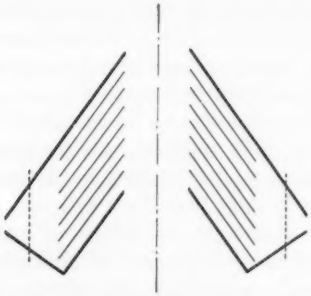

Fig. 6. Peripheral discharge disc bowl

In Type 1, the flow through the nozzles is a function of their size and number and of the liquid head acting on them.

$$Q = K_1 na(2gh)^{\frac{1}{2}} = K_1 na[\omega^2(r_2^2 - r_1^2)]^{\frac{1}{2}} \qquad (53)$$

where K_1 = nozzle coefficient

In Type 2, the same factors apply multiplied by a coefficient which is the proportion of the total operating time that the valves are open.

$$Q = K_1 \frac{t_1}{t} na[\omega^2(r_2^2 - r_1^2)]^{\frac{1}{2}} \qquad (54)$$

where t_1 = valve open time and t = total time.

Since these ports are located outside of the disc stack, they have a negligible effect on the Σ value of the bowl. The nature of the solids when centrifugally compacted becomes of primary importance in the application of centrifuges of this type. The sedimented solids must be of such a nature that they will flow or be extruded through the discharge ports under the pressures available. Their angle of repose must be such that any accumulation of solids between adjacent ports does not build into the disc stack, an effect which will reduce the Σ value of the assembly.

The application of these centrifuges is therefore to systems from which the centrifugally sedimented solids are relatively plastic in nature.

Test data from small-scale equipment are of value in determining the Q/Σ ratio at which satisfactory removal of the solid

phase is obtained within the limits described above. To determine the quantity and physical nature of the sedimented solids the test centrifuge must be operated at a speed to give the same centrifugal force as the final proposed machine.

While the quantity of solids to be removed can be determined quite accurately by this method, no rigorous tests except an actual full-scale run can be applied to determine whether the nature of the solids is such that they will satisfactorily discharge from a peripheral discharge centrifuge of either the nozzle or valve type. There is a considerable background of operating experience with such centrifuges on widely different materials such as yeast, protein, starch, gluten, vegetable oil, mud from wool scouring, clay and tar available to be drawn on, but each new application requires careful individual study with particular reference to the presence and characteristics of minor constituents.

In general, the following considerations apply:

(1) All particles having a diameter greater than half the diameter of the discharge ports must be removed by pre-clarification or straining.

(2) It is not practical to use less than 2 discharge ports in a commercial centrifuge of this type and for many applications the use of 12 or more is desirable to limit the hold-up of solids between ports.

(3) On most applications the smallest practical nozzle size is 0·035 in. diameter which delivers between 60 and 80 gal per h per nozzle from a 6,000 rev/min 19 in. diameter centrifuge depending on the viscosity of the sludge and the density of the liquid.

On certain specific applications such as soybean protein concentration, it may be possible to use smaller nozzles, to 0·031 in. diameter, and on others, such as yeast concentration, special bowls have been developed to return the concentrated solids back towards the centre of rotation, decreasing the net effective head pressure on the nozzles.

For this type of centrifuge, it may therefore be said that small-scale tests will:

(1) Establish the throughput rate, Q, for effective clarification on a given centrifuge rotor,

(2) Indicate the terminal concentration possible in this centrifuge.

(3) Establish the approximate number and size of nozzles required to concentrate the solids into the required portion of the feed stream.

(4) On the basis of the answers to (2) and (3) indicate the relative process advantages of the nozzle and valve type rotors.

Centrifuges with Helical Conveyors

Two modifications of a type of centrifuge that utilizes centrifugal force for both the sedimentation and drainage of the solid particles are shown diagrammatically in Figs. 7 and 8. In these, the sedimented solids are carried to the small diameter end of the

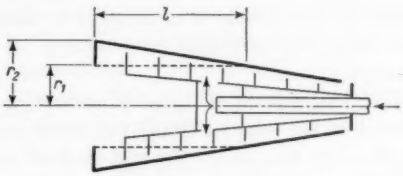

Fig. 7. Conical continuous discharge rotor

rotor by a helical conveyor, which is driven at a speed slightly different from that of the rotor itself. The liquid overflows at the opposite end through ports that may be adjusted with respect to their distance from the axis of rotation for control of the liquid level in the bowl.

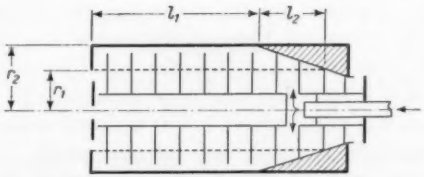

Fig. 8. Cylindrical continuous discharge rotor

In the conical modification, Fig. 7, the sedimented solids are carried up a finite length of 'beach' where they are given an opportunity to drain before being discharged. In the cylindrical modification, Fig. 8, which because of its greater volume has a much larger Σ value for a given length/diameter ratio, the length of time the solids are out of the liquid layer before being discharged is relatively short. In this, the 'drying' is principally effected by compaction of the solids under centrifugal force as they are being scrolled.

For the conical conveyor type of centrifuge the Σ value may be calculated to be

$$\Sigma = \frac{2\pi\omega^2 l}{g}\left(\frac{r_2^2 + 3r_2r_1 + 4r_1^2}{8}\right) \tag{55}$$

where l = length of liquid measured at its surface, r_1 = inside radius of liquid, and r_2 = outside radius of liquid.

From this should be deducted a correction factor of approximately 6 per cent, representing the volume of the conveyor flights in the rotor.

For the cylindrical conveyor type of centrifuge the Σ value can be considered as the sum of the Σ values of a true cylindrical section of length l_1 by equation (33) and that of a conical section of length l_2, it being considered that at the solids discharge end of the rotor a beach is formed to the contour of the conveyor.

The composite formula therefore becomes

$$\Sigma = \frac{2\pi\omega^2 l_1}{g}\left(\frac{3r_2^2 + r_1^2}{4}\right) + \frac{2\pi\omega^2 l_2}{g}\left(\frac{r_2^2 + 3r_2r_1 + 4r_1^2}{8}\right) \tag{56}$$

and again a correction factor of approximately 6 per cent should be deducted for the volume of the conveyor flights.

An examination of Figs. 7 and 8 also indicates that two other factors may decrease the calculated Σ values. In addition to the liquid volume displaced by the conveyor, the sedimented solids that are being conveyed through the rotor displace their own volume of the feed liquid and therefore must be compensated for. In addition, it will be noted that it is impossible to introduce the feed stream at the point where $l = 0$ without washing the solids that are about to be scrolled back into the pond.

Experimental data on a variety of materials have indicated that on the conical conveyor type the actual Σ value approaches

0·62 times the calculated Σ value and on the cylindrical scroll conveyor type the actual Σ value approaches 0·67 times the calculated.

As in other types of solids discharge centrifuges the nature of the sedimented solids is of prime importance. The solids must pack to a consistency that will permit them to be scrolled out of the bowl. In the simplest possible terms, the solids must have a high angle of repose and be of the general nature of material that can be transported by a screw conveyor. Here, as in the peripheral discharge disc machine, tests on a centrifuge that has at least the equivalent of a conveyor is a final requirement since the apparent Q/Σ value for a given degree of clarification will be affected by the presence in the system of any solids that cannot be scrolled.

Laboratory units of this type are available that give good correlation of Q/Σ values with commercial equipment. Since these small units scroll the solids in the same manner as their commercial counterparts, the question of scrollability of the solids is automatically compensated for.

The amount of mother liquid adhering to the discharged solids in a centrifuge of this type appears to follow the general equation

$$\log y = a + bx \qquad (57)$$

where y = proportion of adhering mother liquor, x = reciprocal of the time interval during which the solids are out of the continuous liquid phase, and a and b are constants of the system and the applied centrifugal force, $\omega^2 r/g$.

If two points for a given system are known it theoretically is possible to calculate the complete curve, but this has not been thoroughly explored and resort must usually be made to full-scale operation on commercial size equipment to establish complete data.

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Batch Centrifuge Scale-up for Virus Processing

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Summary. The mathematical relationship between the throughput of a batch centrifuge and its mechanical characteristics has been applied to the scale-up of one of the steps in virus processing for possible application to the production of vaccines and similar antigens. Slurries that contained a test virus were purified in a laboratory centrifuge. The Q/Σ relationship, developed by Ambler, was used to predict the operating conditions of a larger centrifuge in order to achieve the same clarification of slurry as that produced in the laboratory centrifuge. A correlation between conditions of centrifugation and amount of virus removed from the feed to the centrifuge shows that there is a well-defined Q/Σ value at which significant concentrations of the test virus are sedimented.

Introduction

Differential centrifugation has been investigated as a technique for the purification and concentration of viruses for possible application to the production of vaccines or similar antigens.

Vaccines or antigens might be produced by inoculating chicken eggs, containing living embryos, with virus. The eggs are incubated and during this period the virus propagates within the living tissues of the embryo. At the end of the incubation period, the portions of the eggs that are rich in virus are removed from the other components and are ground to a small particle size in a mill in order to release the viruses. Prior to inactivating the viruses for further processing, the resulting slurry may be purified. The slurry is a three-component mixture consisting essentially of viruses, liquid and proteinaceous solids. Laboratory work revealed that centrifugation conditions could be established to purify the slurry, that is, to remove the extraneous protein without removing the viruses, in order that subsequent centrifugation would separate the viruses from the liquid. The initial separation

in the differential centrifugation process is carried out at a relatively slow speed for a short period of time.

The problem then became the scale-up of the output of the laboratory centrifuge to a practical level for production. Expressed in more basic terms, could a sufficient gravitational force be developed in a production type centrifuge to cause sedimentation of the virus as it had in the laboratory centrifuge?

The first step in solving this problem was to define the performance of the laboratory centrifuge in terms of a mathematical quantity, Q/Σ, which can be translated to a larger centrifuge. This approach, developed by Ambler,[1] may be attempted only when the centrifuges are of similar geometric proportions. The term Q represents the throughput while the quantity Σ is defined by the equation: $\Sigma = K\omega^2$, where K is a constant that is fixed for a given centrifuge by its mechanical characteristics and ω is the speed of the centrifuge.

Theoretically, two centrifuges of different sizes but of similar geometric proportions, when operated at the same Q/Σ value, will clarify a given suspension to the same extent. To scale-up from a small centrifuge, s, to a larger centrifuge, 1, the following equation is used:

$$\frac{Q_s}{\Sigma_s} = \frac{Q_1}{\Sigma_1}$$

Once values for Q_s and Σ_s have been established for the desired degree of clarification, the value Σ_1 may be calculated for the desired throughput, Q_1.

In practice, however, results deviate somewhat from those predicted by theory. As capacity is increased, the experimentally determined ratio for various materials shows increased deviation from the calculated ratio. At the lower flow rates there is a better correlation between the observed and the calculated values.

As Ambler observed, for even a reasonably accurate estimation of commercial performance, a laboratory centrifuge must be operated under conditions to give a comparable centrifugal force and, even then, the mathematical relationship between the two centrifuges must be modified by an experience factor.[1]

[1] See Literature Cited.

Experimental Work

In our laboratories these values (Q/Σ) were related to the Sharples Laboratory Supercentrifuge in order to scale-up to the commercially practical capacity of the No. 16 Sharples Supercentrifuge. In each run, 110 ml of slurry, containing a test virus, were centrifuged at bowl speeds from 10,000 to 50,000 rev/min in 10,000 rev/min increments at time periods of 15, 30, 60 and 240 min. The batches of slurry had previously been clarified by centrifuging for five min at a speed of 2,000 rev/min in a refrigerated International Centrifuge, Model PR-1. The total dry solids contents were reduced from 11·5 to 7 per cent. The feed, the sediment, and the bowl fluid were assayed for virus concentration and percentage solids. The average particle size of the test virus was approximately 60 mμ. The amount of virus was determined by the standard bacteriological techniques of inoculating groups of mice intracerebrally with different dilutions of the virus and calculating the concentration of the virus after observing the number of deaths in each group of test animals. Percentage solids were determined by the method of Flosdorf and Webster.[2] During centrifugation, the bowl speed was checked every five min. Bowl temperatures were controlled within the range of 5–15°C by passing refrigerant through a coil within the bowl housing.

The physical measurements of the sedimented slurry under the experimental conditions of centrifugation are given in Table I.

Table I. Bowl fluid (ml) and sediment (g) recovered after batch centrifugation

Centrifugation time (min)	Bowl rotation (rev/min × 1,000) 10		20		30		40		50	
	B.F.	Sed.	B.F.	Sed.	B.F.	Sed.	B.F.	Sed.	B.F.	Sed.
15	98	4·8	96	8·2	93	9·2	93	10·5	94	8·9
30	*	*	93	10·6	*	*	*	*	*	*
60	104	2·4	93	11·8	94	10·8	96	8·8	93	9·4
240	100	1·2	*	*	*	*	93	10·4	96	9·2

* Not investigated

An analysis of the bowl fluid was made in order to determine the percentage of virus that was removed from the slurry under the experimental condition of centrifugation. The results are shown in Table II.

Table II. Percentage of virus removed from slurry as determined by analysis of bowl fluid

Centrifugation time (min)	Bowl rotation (rev/min × 1,000)				
	10	20	30 (%)	40	50
15	0	54	68	78	97
30	*	58	*	*	*
60	40	71	95	99	98
240	15	*	96	99	99

* Not investigated

Increases in time of centrifugation or the development of greater centrifugal forces by increases in bowl speed resulted in the removal of larger amounts of virus from the slurry.

In order to verify the results of the analysis of virus removal from the bowl fluid and to determine if the conditions of centrifugation were harmful to the virus, analysis of the sediment was made. The results are shown in Table III.

Table III. Percentage of virus recovered from sediments

Centrifugation time (min)	Bowl rotation (rev/min × 1,000)				
	10	20	30 (%)	40	50
15	0·4	5·1	65·0	121·0	61·0
30	*	211·0	*	*	*
60	3·1	121·0	528·0	38·4	36·0
240	1·0	*	176·0	188·0	54·0

* Not investigated

The results where recovery is shown to be over 100 per cent may be caused by the virus packing in the sediment and subsequent

difficulty in making uniform re-dispersions for the mouse inoculations. Solid contents of the sediment were determined by the method of Flosdorf and Webster. The results are shown in Table IV.

Table IV. Percentage total dry solids in sediment

Centrifugation time (min)	Bowl rotation (rev/min × 1,000)			
	20	30	40	50
	(%)			
15	12·07	17·64	20·04	*
30	16·90	*	*	*
60	18·90	20·02	23·43	25·81
240	*	23·96	25·05	26·63

* Not investigated

Values of Q/Σ were then calculated for the various conditions of centrifugation. The formula used for the Sharples Laboratory Model Supercentrifuge with one batch of 110 ml of feed is:[3]

$$Q/\Sigma = \frac{4{\cdot}6g}{\omega^2 t} \log \frac{2r_2}{r_1 + r_2}$$

g = 981 cm/sec
r_2 = 2·21 cm (radius of outer surface of liquid layer)
r_1 = 1·78 cm (radius of inner surface of liquid layer)
ω = angular velocity about the axis of rotation, radians per sec
t = time of centrifugation in sec

Table V shows the calculated values of Q/Σ and the total percentage of the test virus recovered in the sediment under the various conditions of centrifugation.

The calculated Q/Σ values were then plotted against the percentage of virus removed from the feed to the centrifuge (Fig. 1). The plot indicates graphically that there is a break point in the curve at about $0{\cdot}5$ to $1{\cdot}0 \times 10^{-8}$ cm/sec where there is no significant increase in virus recovery for a decrease in Q/Σ. Although there is some virus recovery from the sediment at every condition of centrifugation, as shown in Table III, significant concentrations of virus (90 per cent or more) are not removed from

Table V. Summary of virus recovery and Q/Σ values for the Sharples Laboratory Centrifuge

Centrifuge, rev/min × 1,000	Conditions, min	Virus recovered from sediments (%)	Q/Σ values (cm/sec) × 10^{-8}
10	15	0·4	20·35
10	60	3·1	5·08
10	240	1·0	1·27
20	15	5·1	5·09
20	30	211·0	2·54
20	60	121·0	1·27
30	15	65·0	2·26
30	60	528·0	0·56
40	15	121·0	1·27
40	60	38·4	0·32
40	240	188·0	0·08
50	15	61·0	0·81
50	60	36·0	0·20
50	240	54·0	0·05

the feed until a Q/Σ value of 0·5 to 1×10^{-8} cm/sec is reached. It might be noted here that the centrifugation time of 30 min was run at only one bowl speed. The calculated Q/Σ indicated that the point would fall on the threshold of efficient virus separation from the feed.

A correlation between conditions of centrifugation and the effects of imposing these conditions on the feed is shown by a graph of Q/Σ versus percentage of total dry solids in the sediment (Fig. 2). The relationship between virus concentration in the sediment and centrifuge bowl speed is given in Fig. 3.

There is an interesting relationship between the amounts of virus in the bowl fluid and in the sediment and the conditions of centrifugation. When Q/Σ is $1{\cdot}0 \times 10^{-8}$ cm/sec or less, the majority of the virus is removed from the bowl fluid. The virus is recovered from the sediment. If the virus was not recovered in the sediment it could be concluded that centrifugation conditions were too severe and the viruses were being killed or so severely

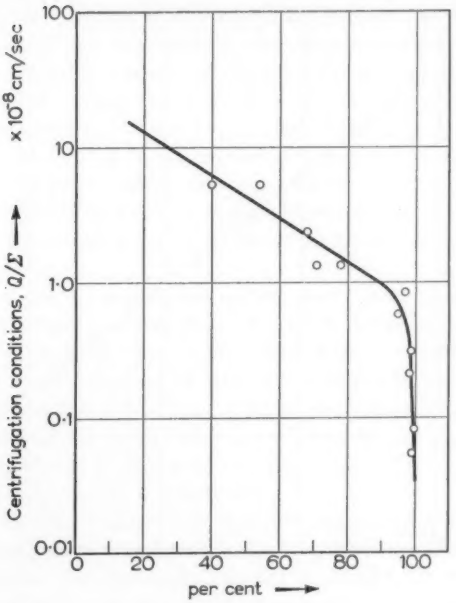

Fig. 1. Influence of centrifugation conditions, expressed as Q/Σ, on percentage of virus removed from the feed

packed that a precise assay could not be made. The apparent decrease in virus recovery under the more severe centrifugation conditions as shown in Table III indicates that values of Q/Σ below $0{\cdot}5 \times 10^{-8}$ cm/sec may have been harmful to the test virus or to the reliability of its assay.

The limiting values of Q/Σ established in this study may then be used in calculating operating conditions for the larger centrifuge in order to achieve the same degree of purification and concentration of the test virus. For example, if it is desired that at least 90 per cent of the virus be removed from 500 ml of slurry in a Sharples No. 16 Supercentrifuge, the corresponding Q/Σ value for the correct operating conditions will be $1{\cdot}0 \times 10^{-8}$ cm/sec (Fig. 1). After substituting into the formula the new radial distances of the

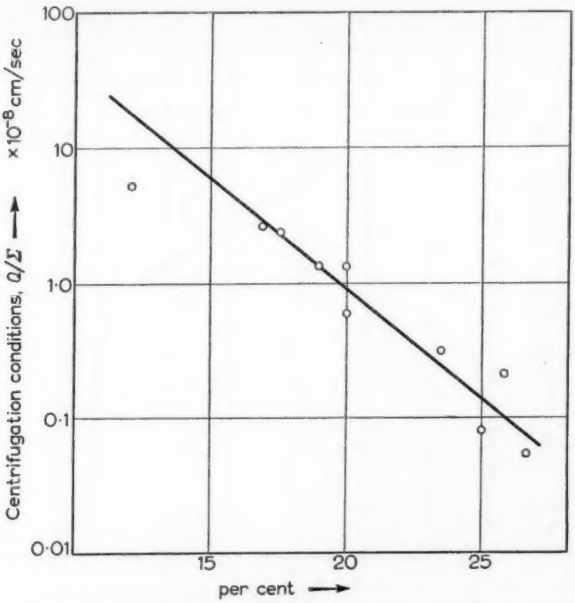

Fig. 2. Influence of centrifugation conditions, expressed as Q/Σ, on percentage of total dry solids in the sediment

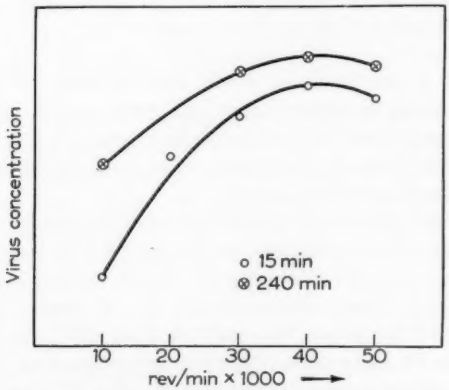

Fig. 3. Influence of centrifugation conditions on concentration of virus in the sediment

inner and outer surfaces of the liquid layer from the axis of rotation, the answer for the correct operating conditions will be in terms of the angular velocity and time of centrifugation; that is, $\omega^2 t = 99{\cdot}4 \times 10^8$. Since ω is limited by the maximum recommended bowl speed of 17,000 rev/min, the minimum time of centrifugation for the 500 ml batch will be 52·5 min. Theoretically this will result in approximately the same virus removal as a 110 ml batch that is clarified in the laboratory centrifuge operating at a speed of 30,000 rev/min for 34·5 min or at 40,000 rev/min for 19·5 min.

Several batches of uninfected milled slurry were clarified in the Sharples Laboratory Centrifuge at Fort Detrick. Using the method of calculating that is described above, predictions were made of the total amounts of sedimentable solids that would be obtained with a Sharples No. 16 Supercentrifuge. The experimentally determined results were lower than the predicted results by about 25 per cent for three of the four runs. The results of the fourth run were exactly as predicted.

Although the Q/Σ relationship in batch centrifuge scale-up is not as accurate as might be desired, the application of this mathematical method has been found to be useful in establishing operating conditions for one of the steps in virus processing.

Acknowledgements

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The Evaluation of a Process for the Preparation of Co-enzyme A from Yeast

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Summary. A process is described for the extraction of co-enzyme A from dried yeast. It involves *cold water* extraction, charcoal adsorption followed by pyridine elution, preparation of an acetone powder, co-precipitation as the double cuprous complex with glutathione and removal of the glutathione by means of an ion exchange resin.

Improvements over previous processes include use of drum dried yeast plus cold extraction resulting in easy filtration and increase of throughput by a factor of three to five, together with the elimination of the ion exchange step formerly thought necessary to remove zinc before the co-precipitation stage. Under the best conditions yields represent 30% recovery of the co-enzyme A in the yeast, the product being 90% pure.

Since the discovery of co-enzyme A by Lipmann in 1947, successive laboratory workers have developed processes for its preparation. Lipmann, Kaplan, Novelli, Tuttle and Guirard (1947) first isolated a concentrate from pig liver by successive precipitation with mercury, lead and silver salts, and this was elaborated by the same workers in 1950. Lipmann *et al.* (1950) also prepared it from a bacterial culture fluid by selective adsorption on charcoal.

The first preparations from yeast appear to have been made by Buyske *et al.* (1951) at Wisconsin using barium precipitation, ion-exchange chromatography and activated charcoal, all of which gave some degree of purification, but poor stage yields. An important advance was made by the same laboratory (Beinert *et al.*, 1952, 1953) when it was discovered that co-enzyme A could be co-precipitated together with glutathione by the use of cuprous sulphate, a precipitant already well known for the precipitation of glutathione alone (Hopkins, 1929).

Stadtman and Kornberg (1953) reported a simple two-stage process from yeast which depended on charcoal absorption followed

by fractional elution from an ion exchange resin (Dowex 1×2). Unfortunately, under their conditions low degree of cross-linkage of the resin is necessary in order to obtain good capacity for the large co-enzyme A molecules. This process, therefore, is very difficult to manage on a large scale, and the degree of purification of the co-enzyme A tends to be poor and erratic.

The Wisconsin process, therefore, seemed the most promising when it was decided to attempt the preparation of this co-enzyme in Great Britain, and it was decided to evaluate and improve this process with the materials and plant available locally.

Methods

Co-enzyme A was estimated by the phosphotransacetylase method of Lipmann, Stadtman and Novelli (1951) using *E. coli* grown anaerobically as a source of the enzyme. The method was developed to make an assay suitable for the estimation of large numbers of plant samples (Reece, 1958). The procedure allows seven samples to be assayed simultaneously at short notice and by one man, together with the necessary blanks and calibration measurements.

Sulphydryl was estimated by the nitroprusside reaction, using Von Korff's modification of the method of Gruenert and Philips (1951).

Glutathione was determined by the Alloxan–305 method (Patterson, Lazarow and Levey, 1949) or by titrating the sulphydryl group with iodine.

Pyridine in aqueous solution was estimated by direct titration with standard acid (Fowler, 1948).

Evaluation Problems

The process as finally worked out and the quantities involved on the batch size adopted are summarized in Fig. 1 which indicates the steps from the raw material to the final product. It resembles the Wisconsin process but departs from this in important particulars.

A consideration of the Wisconsin process in the light of biochemical laboratory preparations in general indicated that the most difficult problems would be:

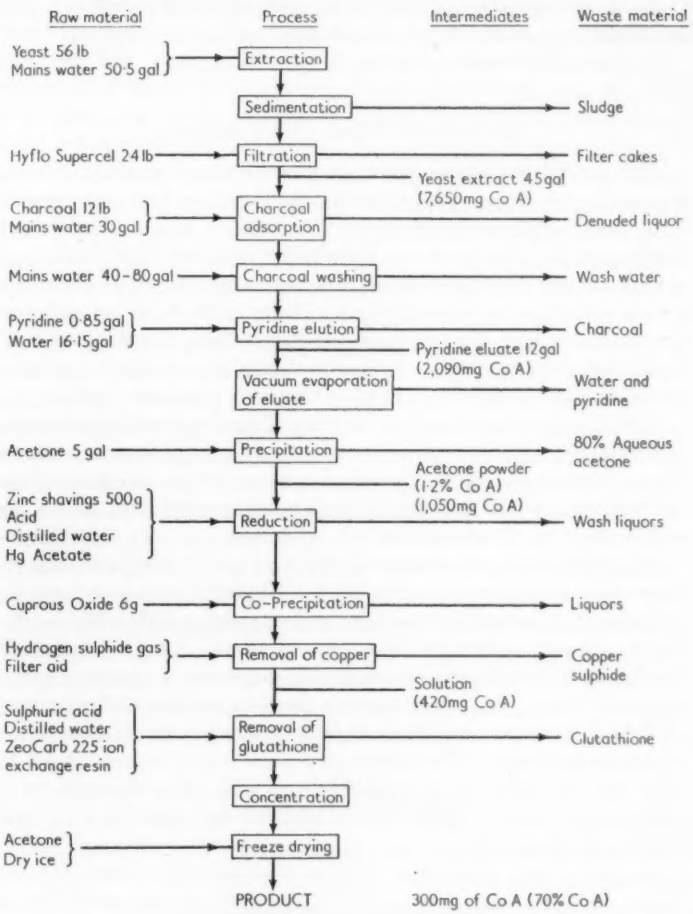

Fig. 1. Flow sheet

(1) Finding suitable sources of raw materials, particularly yeast and charcoal.

(2) Preparing and filtering the yeast extract.

(3) Evolving modifications to the process which would eliminate or simplify as many stages as possible.

These will be briefly discussed.

Source of Yeast and Charcoal

The yeasts available were various top and bottom brewing yeasts, bakers' yeast either pressed or dried at low temperature, from the Distillers Company (D.C.L.) and a drum-dried poultry feeding yeast, also from the Distillers Company. This is a yeast grown on residues from whisky production. In the early stages of the investigation the drying had been carried out on a directly heated drum and the product was fairly dark in colour and of a hard, flaky consistency. Later, a steam-heated drum was introduced which resulted in a lighter coloured, powdery product.

Initial experiments, using the usual boiling and rapid cooling technique for extracting labile materials from yeast cells, followed by estimation of the co-enzyme A content of the clarified extract, showed that the D.C.L. drum-dried yeast was the best source, and subsequent work was carried out with this.

The difficulties encountered by Beinert *et al.* (1952) with charcoal were also encountered in this work. British types were narrowed down to eight possible products, and on testing with a filtered hot-water extract of yeast, only one of these, Sutcliffe–Speakman Carbon 132, was found to combine the necessary qualities of low-pressure drop with yeast liquors, good adsorption of co-enzyme A from liquor and acceptable regain of co-enzyme on elution with aqueous pyridine. The properties were by no means ideal, but this was the best type available and was used throughout the subsequent work. Each batch had to be checked and many were unsatisfactory.

Extraction and Filtration

Initially the usual biochemical laboratory method, also used by Beinert *et al.* (1952), was employed to make a co-enzyme A containing extract. The dried yeast was added to boiling water, the temperature brought back to 95–100°C with live steam and maintained at that for 5 min. It was then cooled rapidly below 40°C

by the addition of crushed ice and clarified. Beinert *et al.* used centrifugation but this is clearly out of the question for medium sized batches, since about 20 per cent of the volume is solids. Filtration of these slurries presents very great difficulties because of the small size of the yeast cells and the extreme compressibility of the cake.

Similar slurries result when yeast is cytolysed by other methods such as treatment with acid alcohol–ether mixtures. Satisfactory filtration of all types can be accomplished by the use of the correct proportion of the right type of filter aid and meticulous attention to detail, and it is hoped to report on this aspect of the work at a later date.

For the present process, the solution of the problem was found in an unexpected direction. Attempts were made to obtain a more easily filterable slurry by reducing the temperature of extraction. It was then found that the amount of co-enzyme A extracted actually improved on lowering the temperature by steps down to that of the mains water when 100 per cent extraction was obtained. Five to ten minutes stirring were sufficient to complete the extraction.

The subsequent handling of the material was considerably simplified because, during such a short extraction at the low temperature, a large proportion of the hard flakes remained intact and sedimented to a compact volume. The filtration was thus fairly good using approximately 4 per cent of the liquid weight of a filter aid with particles of approximately the size of the yeast particles (Hyflo Supercel—Johns Manville).

Process Modifications

Apart from the quite large change entailed in going from hot extraction and centrifugation to cold extraction and sedimentation, it was found advantageous to introduce other modifications into the Wisconsin process. Thus, Beinert *et al.* (1952) had seemed to prefer solvent extraction with carbon tetrachloride for removal of pyridine from the charcoal column eluate before concentration rather than to proceed direct to concentration *in vacuo*. They mention that too long a delay in removing the pyridine may result in lowered yields. This is understandable in view of the

well known catalytic effect of pyridine on the hydrolysis of other phosphate compounds, e.g. acetyl phosphate (Koshland, 1951). It was found in the present work that, with the efficient climbing film vacuum evaporator which was specially designed for the purpose, the most efficient procedure was to remove the pyridine during the process of concentration. The evaporator worked at a vacuum of 28·5 in. Hg., and the temperature remained below 30°C. Under these conditions the loss of co-enzyme A was found to be negligible.

Beinert *et al.* (1952) recommend that, after the acetone powder has been made this should be re-fractionated on charcoal, including a mild alkaline wash with 0·02N sodium bicarbonate solution, and a second acetone powder prepared. The present investigation confirmed that this procedure leads to a 2- to $2\frac{1}{2}$-fold increase in purity, but with the charcoal available resulted in poor recovery of co-enzyme A (not more than 40 per cent over the stage). It was only used, therefore, when very impure first acetone powders were obtained.

At the co-precipitation stage, Beinert *et al.* (1953) introduced a reduction step to economize on glutathione, part of which is otherwise consumed in reducing disulphides; they used a Jones reductor (amalgamated zinc) and removed the zinc ions by passing the liquors through a cation exchange resin column (Dowex 50). Unfortunately this also removes all the glutathione which has been extracted from the yeast and concentrated along with the co-enzyme A, and they were therefore obliged to add back all the glutathione required for the subsequent co-precipitation.

In the present work, removal of the zinc ions was found unnecessary: indeed, in most runs, their presence was found beneficial. Thus, not only is the ion exchange step (and the subsequent regeneration of the resin) eliminated, but there is a great saving in glutathione—an expensive commodity. In many runs it was not necessary to add glutathione at all, and in the remainder, only small amounts—not more than one-fifth that specified by Beinert *et al.* (1952).

Processing

The raw material was poultry feeding dried yeast (Distillers Co.). 50·5 gal of cold mains water was pumped into an extraction

vessel, the agitator started and 56 lb of yeast shovelled in. After 10 min the slurry was pumped into a 100 gal settling pan and allowed to settle for 60 min. Extraction was carried out in a 75 gal E.M.P. vessel but any agitated vessel is suitable. To allow sedimentation to be completed in a reasonable time a shallow pan is needed. An improvement in the yield can be made by washing the sludge with a further 10 gal of mains water.

The supernatant liquor, removed by a simple decanter (Fig. 2),

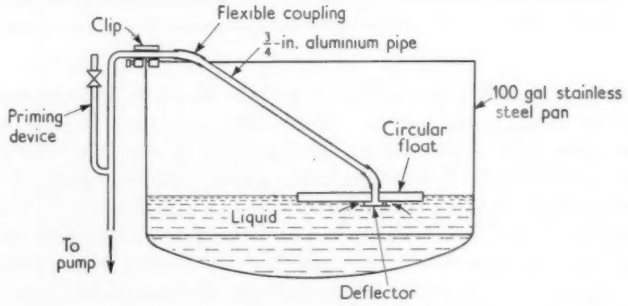

Fig. 2. Supernatant decanter

was then filtered on a plate and frame filter press using filter aid. Maximum recommended amounts of pre-coat were used, 10 lb/100 ft² and filter aid added at the rate of 4 per cent of the liquid weight. Filtration is best carried out at very low pressure; best results were obtained at pressures below 7 lb/in². With higher pressures rapid blinding occurs. The filter press used was a 23 ft² total area, hard rubber, plate and frame press of the non-washing type. It consisted of 10 frames, 12·6 in. square and ¾ in. in depth. Three 20 gal vessels piped together formed the filtration feed vessels and the contents were passed to the press via a centrifugal pump, rate 600/200 gal/min at 5/35 ft head. This pump was wired in series with a variable transformer which gave control of pump speed. A single clip-on agitator which could be fitted to any of the three filter feed vessels was needed to prevent the filter aid from settling out (Fig. 3).

Filtrate, a sparkling, yellow liquor containing 37·5 ppm of

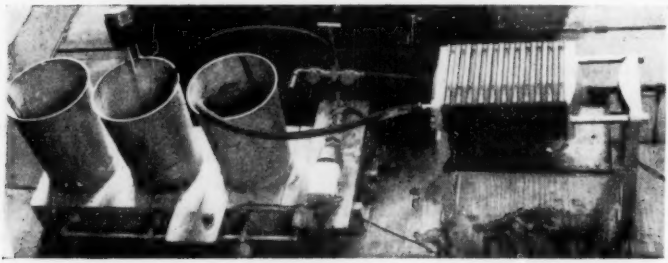

Fig. 3. Filter press and filtration feed vessels with clip-on stirrer

co-enzyme A was then carried in mobile 40 gal containers to the charcoal adsorption column. This consisted of a 6 ft long, 6 in. diam glass pipe section fitted with a packing support. 12 lb of charcoal (Sutcliffe Speakman Ltd., grade 132) was poured into 4 gal of water in the column and allowed to settle out. By means of agitation and back-washing a closely packed and air-free bed of charcoal was obtained. Through this the filtrate was passed at the rate of 12·5–15 gal/h and the effluent discharged to waste.

It was essential to wash the saturated charcoal thoroughly. Up to 80 gal of mains water was required, passed at the rate of 5 gal/h. Elution of the co-enzyme A active material was achieved with 17 gal of 5 per cent pyridine in water. The activity came off as a single peak which coincided with the sulphydryl material and the depth of colour of the eluate (Fig. 4).

Pyridine was removed from the eluate and the volume reduced simultaneously by means of a continuous vacuum evaporator. The all-glass climbing film evaporator had a capacity of 11 gal/h at a vacuum of 28·5 in. Hg. The concentrating liquor passed round a closed circuit as pyridine and water vapour were removed continuously. Before the evaporator was shut-down processing was carried on for 3 min without feed to clear the remaining pyridine in solution.

Concentrated liquor, freed from pyridine, was made into an acetone powder; the liquor, acidified with sulphuric acid to pH 2·5, was precipitated by pouring into five volumes of cold acetone. This product could be collected in one of two ways, either by standing overnight, when the clear supernatant liquor could be

decanted easily and the precipitate removed, or by passing the slurry through a laboratory size, Sharples long-bowl centrifuge working at a speed of 12,500 rev/min. In both cases the product

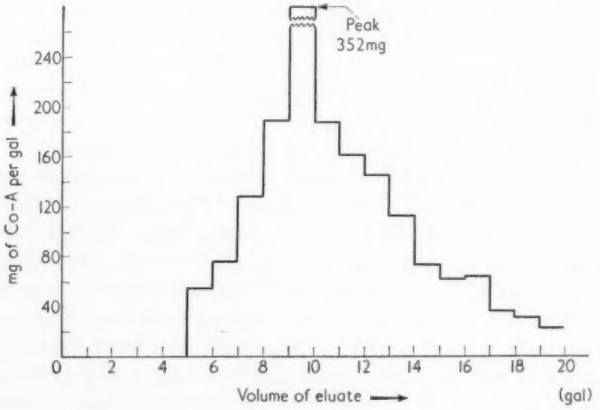

Fig. 4. Co-enzyme A elution from charcoal with 5 per cent aqueous pyridine

is glutinous and often becomes tarry when exposed to air. This material could be reduced to a dry, free flowing powder, however, by repeated trituration with fresh dry acetone.

The next processing stages were all carried out in the laboratory because the quantities involved were small. The principal operation was the co-precipitation of the co-enzyme A with glutathione and cuprous oxide. The reduction process before the main precipitation was carried out in a Jones reductor prepared by packing a column, 7 cm diam and 65 cm deep, with pure zinc turnings. This was prepared by first filling the column with 1 per cent mercury acetate solution for 10 min—to amalgamate the zinc—washing the zinc thoroughly with distilled water and then filling the column with 1N sulphuric acid for 5 min to activate the zinc. The liquors, made 0·5N with respect to sulphuric acid, were passed through the column over a 30 min period.

Product from the reductor was placed in a precipitation beaker,

the acidity corrected to 0·5N and then a cuprous oxide suspension added until a flocculated precipitate formed. The precipitate was collected by allowing it to settle and decanting off the liquor. This operation is difficult because the precipitation does not follow stoichiometry and experience is needed to judge the correct amount of oxide needed. If too much cuprous oxide is added the precipitate goes back into solution. This operation is described in detail in the preparation of glutathione (Hopkins, 1929).

The precipitate was washed with distilled water until sulphate free and the copper removed by gassing a suspension in distilled water overnight with hydrogen sulphide. The glutathione and co-enzyme A remained in solution. The copper sulphide was removed by filtering on a small Buchner filter through a layer of diatomaceous earth (Filter-cell, a product of Johns Manville Ltd.). The clarified liquor could be freeze-dried directly if a product of 8–12 per cent co-enzyme A content was sufficient, but normally the glutathione was removed by passing a solution through a bed of Zeocarb 225 resin (Permutit Ltd.) packed in a glass tube 3 cm diam to a depth of 25 cm. The liquid was passed through the resin in 30 min or less to avoid losses.

The resulting solution of co-enzyme A was reduced in volume in a laboratory climbing film vacuum evaporator and finally freeze-dried either in ampoules or in bulk in a flask. The product is a white amorphous material which is deliquescent. Assays show the product can range from 60 to over 90 per cent co-enzyme A content.

Discussion

This process can be regarded as successful insofar as it can, with attention to detail, be used for the production of co-enzyme A of 90 per cent purity on the gramme scale. However, unless the yields are to be poor careful batch-testing of raw materials is necessary; both yeast and charcoal are extremely variable. Many batches of yeast either have low co-enzyme A contents or give poor extractions. Acetone powders containing less than 1 per cent co-enzyme A may then be obtained. Impure acetone powders yield very little co-enzyme A at the stage of co-precipitation with cuprous sulphate and glutathione. The reason for this is not

understood, but may be connected with the solubility product of the complex. Even with good acetone powders, yields at the copper precipitation stage tend to be not more than 50–70 per cent.

Many batches of charcoal show poor absorption of co-enzyme A or low regain or both. Testing can only be done by running a miniature co-enzyme A preparation.

If untested samples of yeast and charcoal taken at random are used, the results reported in Fig. 1 are obtained which represent a typical run under such conditions. The overall recovery represents 4 per cent of the co-enzyme A in the original extract at a level of purity of 70 per cent. By batch-testing the raw materials and attention to detail, with careful analytical checks at most stages, this can be raised to nearly 30 per cent recovery at 90 per cent purity. This is approximately that achieved by Beinert *et al.* (1952) on the laboratory scale (12 lb of yeast) and the purity is somewhat better (Beinert *et al.* give 83 per cent).

It is difficult to compare the overall yield attained in the present work with that of Beinert *et al.* (1952, 1953) at Wisconsin, as they do not give any figures for the co-enzyme A content of their yeast. If the present experiments are taken as an indication, the overall yields attained in this work are better. Cold extraction gave 100 per cent of the co-enzyme A in the yeast, but hot extraction only about 50–60 per cent in various experiments.

The filtration stage has been greatly improved so that there are negligible losses here. Under the best conditions, the copper stages and subsequent recovery have also been improved. It might have been expected, therefore, that recovery from the extract in good runs should have been rather better than those of Beinert *et al.* rather than of about the same order. The answer undoubtedly lies in the charcoal stage. In the present work it has never proved possible to obtain a charcoal which would take up more than 50 per cent of the co-enzyme A in the crude filtrate nor to show a regain of more than 50 per cent of that adsorbed when eluted with aqueous pyridine. The overall yields of this stage of 25 per cent, therefore, are to be compared with overall yields of 50–60 per cent from the charcoals available to the American workers.

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An Automatic Apparatus for Extraction of Cell Suspensions by Repeated Freezing and Thawing

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Summary. An account is given of the construction and operation of a fully automatic apparatus in which the material is kept under agitation in a closed container alternately surrounded by hot and cold alcohol supplied from separate baths. The apparatus has the following applications in microbiology and cell physiology:

(1) Extraction of micro-organisms and cell tissues by repeated freezing (F) and thawing (T) at variable temperatures and lengths of FT cycle. Extraction can be controlled automatically or manually. As a practical example of the use of the apparatus a description is given of the extraction of living virulent *S. typhi* bacteria.

(2) Studies of the reactions of living micro-organisms and tissues under varying physical conditions (e.g. high- and low-temperature resistance experiments, osmotic studies, thermal synchronization of bacterial cultures, etc.).

(3) If required, the normal gas mixture in the residual air space can without difficulty be replaced by other gases or mixtures such as CO_2 or N_2.

Introduction

A procedure based on the extraction of micro-organisms by repeated freezing (F) and thawing (T) is sometimes used in microbiology, the effect being supported by osmotic disproportion. This principle was patented by A. Guerber (1899–1901) in connection with his method for producing concentrated solutions of heat coagulable protein material.[1] The FT procedure was later used in microbiology by several workers to extract soluble antigens and toxic materials from a number of different micro-organisms.[2–16] The advantages of the procedure lie in its simplicity and in the small hazards it involves of modification of sensitive materials, as may happen when using chemicals.

Assuming that the proportion of cell material to distilled water is in a qualitatively favourable relationship for processing of the material, the following additional conditions should be fulfilled:

(1) The osmotic effect is likely to be best utilized if the cell material is evenly distributed in the liquid phase. The suspension should, therefore, be prevented from sedimenting in the unfrozen state, and some kind of agitation will accordingly be necessary.

(2) Provision must be made for measuring and recording the temperature of the material throughout the entire process. In many bacteriological studies it is important to be able to vary continuously the maximum and minimum temperatures and also the duration of the hot and cold periods.

These requirements can be fulfilled by manual methods, but would be attended by practical difficulties involving loss of time and risks of various kinds. For example, the risk of infection in manual work on pathogenic micro-organisms should not be under-rated. Further problems arise if the suspension is, for example, to be kept in a nitrogen atmosphere.

The treatment of the cell suspension should naturally take place in a closed system. Under the manual procedure adopted hitherto the material was placed in a centrifuge tube or similar vessel which was immersed alternately in a hot and in a cold liquid bath, and with such a vessel, which must be closed with some form of lid or plug, it is very difficult to arrange for agitation of the material in a practical manner. It is likewise difficult to obtain reliable measurements of temperature which implies in reality a factor of uncertainty which makes it impossible to reproduce the tests exactly. The greatest difficulty in manual work of this kind is the waste of manpower, since each complete operation takes anything from 8 to 72 h.

To circumvent these problems as far as possible a fully automatic apparatus has been designed which has proved to satisfy the aforementioned requirements.

Description of Apparatus

The apparatus is shown in a schematic form in Fig. 1. It consists of three main components, viz. a *cold bath* 1, a *hot bath* 2 and the *processing vessel* 3, with the necessary instruments and

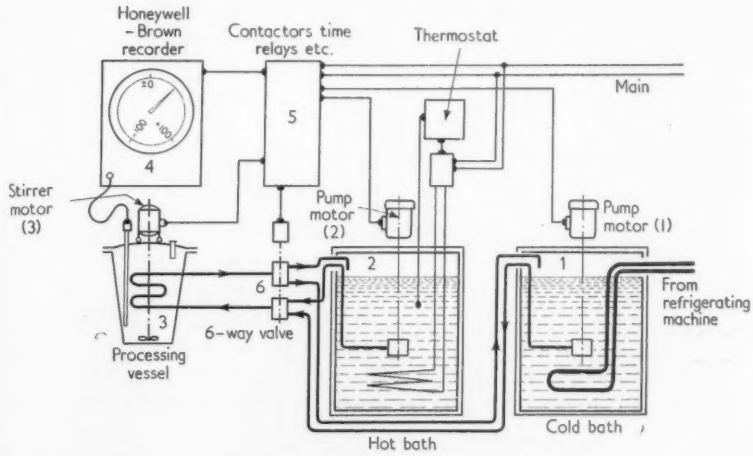

Fig. 1. Schematic diagram of Freeze–Thaw apparatus.

Fig. 2. Front view of Freeze–Thaw apparatus. The lid of the processing vessel, with stirrer motor above it, is seen (left) in front of the Honeywell–Brown recorder. The two pump motors for hot and cold baths are to the right.

automatic equipment. The cold bath which, like the hot bath, has a total capacity of about 100 l., contains about 75 l. of ethyl alcohol which is cooled by a 3-stage refrigerating machine with a calculated capacity of 2,000 kgcal/h at a vaporization temperature of $-70°$. The hot bath likewise contains about 75 l. of alcohol and is heated by a 2-kW electric heater, being kept at an optional constant temperature (between room temperature and max $+60°$) by means of a thermostat. The vessel in which the cell suspension is kept during freezing is called the processing vessel. The 3,000 ml vessel used in our experiments is shown in Figs. 3, 4 and 5: Figs. 4 and 5 show how the vessel is built into the apparatus. It is easily removable and, if desired, can be replaced by a vessel of 50 ml, 100 ml or other volume. It is made of acid-proof stainless steel and is double-jacketed. The hot or cold medium passes between the inner, corrugated jacket and the

Fig. 3. The processing vessel showing the deeply corrugated inner jacket and the finned tubes with the shaft of the removable turbine stirrer passing up through the centre. Note the rubber ring which forms a seal between the vessel and the lid.

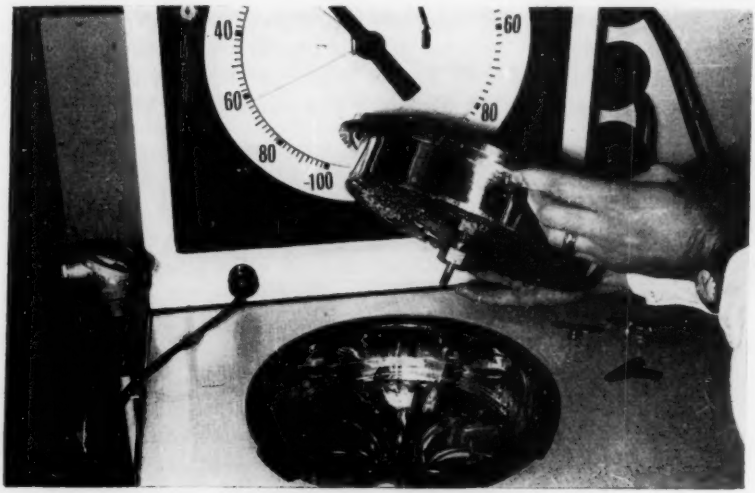

Fig. 4. The processing vessel with lid removed.

outer jacket. The inner cavity is filled with finned tubes (see Fig. 3). The total heat-conducting area of the 3,000 ml vessel is 5,000 cm^2, and no part of the sample is more than about 7 mm from a heat-conducting surface. The vessel is fitted with stirrers which pass through the lid, a seal being provided by a Teflon gland. The thermocouple of the Honeywell–Brown recorder 4 also passes through the lid. Two other openings through the lid are used for filling and emptying the vessel or for sampling in the course of an experiment. When not in use these openings are sealed by a rubber gasket in the lid. To empty the vessel, a stainless steel tube is lowered through one of the openings and the contents are forced out by compressed air or withdrawn by suction. On the completion of an experiment the vessel can be sterilized *in situ* by blowing in steam through one of the openings, the condensate so formed being removed through the stainless steel tube. To eliminate the risk of any surviving micro-organisms, we have generally kept the vessel at 100° for half an hour. After work with sporing material the vessel is placed in an autoclave for sterilization at 120°.

The operation of the machine during a complete hot and cold cycle is as follows. Assume that the temperature in the vessel is rising: from the hot bath alcohol is pumped through one-half of the sliding valve 6 (Fig. 1) to the processing vessel and back to

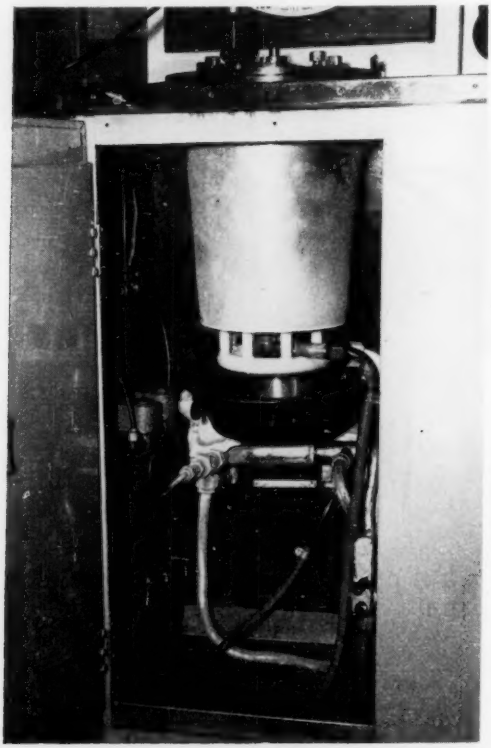

Fig. 5. The processing vessel at the start of a freezing cycle. Note the frost on the vessel.

the hot bath via the other half of the valve. When the culture suspension has reached the upper temperature limit set on the Honeywell–Brown recorder, a relay 5 is actuated which disconnects pump 2. The electrically controlled valve 6 cuts off the supply between the processing vessel and the hot bath and

switches it instead to the cold bath. Pump 1 now immediately starts. The machine is also equipped with a time relay which delays the operation of the valve after the stopping of pump 2 by up to 60 sec. During this time the hot alcohol is removed from the processing vessel by automatic application of compressed air before the cold alcohol is pumped in. At this stage the refrigerating machine has usually reduced the temperature of the alcohol in the cold bath to about $-75°$. When the cold alcohol enters the outer compartment of the processing vessel, the temperature of the culture suspension falls rapidly. A crust of ice almost immediately forms on the inner walls of the vessel. With the location of the thermocouple used in our experiments, the culture suspension was completely frozen when the Honeywell–Brown recorder showed $-15°$. Just previously (at $-12°$) an impulse from the recorder switches off the stirrer motor 3, but the construction of the motor is such that it suffers no serious damage if left switched on for a short time while the propeller is ice-bound. When the lower temperature limit has been reached, the Honeywell–Brown recorder switches off the pump motor 1. Compressed air can now be blown through the system. The valve switches over and the hot bath pump 2 starts. The culture suspension adjoining the heat-conducting surfaces immediately starts to melt. In practice the Honeywell–Brown recorder has indicated about $+30°$ before all ice in the vessel has melted. At this temperature, therefore, the stirrer motor is automatically switched on.

The operation of the machine has now been followed through a complete freezing and thawing cycle (a full FT cycle). Sectors of the Honeywell–Brown graph paper are shown in Figs. 6 and 7. Fig. 6 shows the temperature conditions in the processing vessel when it is run empty: Fig. 7 shows the conditions when it is filled with 2,000 ml of fluid. The cycles are of course shorter when the vessel is empty.

To illustrate the operation of the apparatus we take the following extraction experiment.

175·0 g of wet bacterial mass, corresponding to 31·8 g dry weight (after freeze-drying) of the *S. typhi* strain 52/A/48 (Vi, 0 : 9, 12, h) was suspended after washing in a physiological solution of NaCl in 525 ml of distilled water. The suspension was then subjected to 20 FT cycles and centrifuged; the water phase was removed as

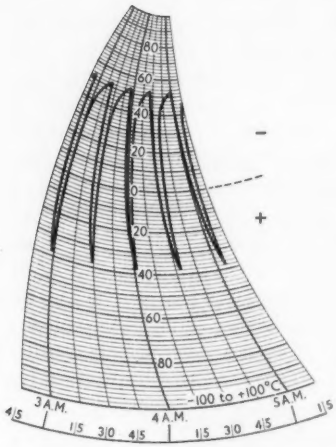

Fig. 6. Record of temperature in empty 3,000 ml processing vessel. Example: temperature range −54 to +38°. Honeywell–Brown recorder.

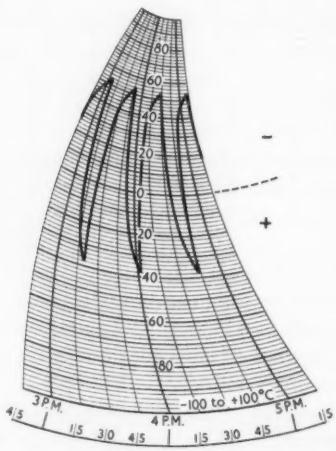

Fig. 7. Record of temperature in 3,000 ml processing vessel containing 2,000 ml suspension. Example: temperature range −54 to +38°. Honeywell–Brown recorder.

extract No. 1 and the bacterial phase re-suspended in 525 ml of distilled water and subjected to the same FT procedure 20 times. Renewed centrifuging yields extract No. 2, after which the same procedure is repeated a third time under the same conditions.

The temperature range for this example was −54 to +38°. To obtain a rough idea of the death-time curves, samples were collected under sterile conditions from the first to last FT cycle, each sample consisting of 0·1 ml of the suspension and being collected at the maximum temperature +38°. The material was transferred with a spatula to a horse-blood Petri dish in order to calculate and control any survival quotient in the suspension during the FT process.

After the completion of the FT process we accordingly have extracts 1, 2 and 3, and the bacterial residue.

In this experiment with a maximum temperature of +38° no further growth of bacteria was found after 37 FT cycles.

The sum of extracts 1, 2 and 3 on a dry substance basis is about 25 per cent of the dry weight of the original quantity of bacteria.

Table I

Extract No.	Yield from original 31·8 g dry wt		pH	Nitrogen % total	Nitrogen % formalin titration	Molisch react. for carbohydrate	Heller react. for proteins
	g	%					
1	3·51	10·91	5·94	9·65	0·0123	+++	?
2	2·61	8·20	6·07	11·40	0·0129	+	−
3	1·74	5·47	6·09	11·00	0·0130	±	−
	7·86	24·58					

Applications

The apparatus can be used in microbiology and to some extent in cell physiology for the following purposes:

(1) Freezing–thawing within determinable temperature ranges for extraction of micro-organisms and cell tissues in any desired atmosphere (e.g. N_2, CO_2, etc.).

The design of the apparatus permits variable settings of minimum and maximum temperatures within the limits -80 and $+60°$, the latter depending on the alcohol medium used, and also conditional variations in the lengths of the FT cycles. If the intervals are to be varied during an FT process, they can be regulated manually by means of special switches.

(2) Studies of the reactions of various (living) micro-organisms or tissue cultures under different physical conditions—such as rhythmically varying temperatures, autogenous, metabolistic changes in pH during the growth period—or the reactions of culture suspensions and certain biochemical processes (antigen and toxin development, etc.) under different osmotic conditions, etc., at constant or varying temperature within the maximum ($+60°$) and minimum ($-80°$) limits permitted by the apparatus. It can also be used for producing thermally synchronized bacterial cultures.

Acknowledgement. This project was made possible by research grants from The National Association of Sweden against Tuberculosis and Other Endemic Diseases. We wish to extend our especial thanks to the Association for this assistance and for the confidence reposed in us.

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Note

The Interaction of Unsaturated and Saturated Steroids in the Production of Δ1,4-androstadienedione with *Fusarium caucasicum*

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Vischer and Wettstein[1] converted progesterone into Δ1,4-androstadienedione (Δ1,4) in 80 per cent yield with *Fusarium solani*. With *Fusarium* strains at our disposal the course of oxidation was not uniform. Using the experimental technique described by the Swiss authors, in addition to Δ1,4, *Fusarium caucasicum* produced Δ4-androstendione (Δ4) and Δ1-testololactone from progesterone in the course of a conversion period of 24 h. We have investigated the effect of steroid compounds of different structures on each other's oxidizability in cultures of *Fusarium caucasicum* and we have looked for a method by which it is possible to obtain a uniform product from mixed substrates.

F. caucasicum was grown on a shaking machine in 100 ml portions of medium containing peptone, glucose and corn-steep-liquor in 500 ml conical flasks. The various substrates were added to the cultures aged 24 h, the Δ1,4 formed was estimated concurrently by the quantitative Kober–Haenni reaction. The other products were detected and identified by paper chromatography using a propylene–glycol–carbontetrachloride–methylcyclohexane solvent system.[2] Fig. 1 shows that progesterone was converted (50 per cent yield) into Δ1,4; at the time of peak production Δ4 and Δ1-testololactone were also found by paper chromatography.

Allopregnanedione gave better yields of Δ1,4 which in this case was used up more slowly in the metabolic process, also its

production peak was reached later. When progesterone and allopregnanedione were added together to the culture, the production peak of Δ1,4 was reached later. Also it was higher than the superposition of the two previous production curves would suggest and this higher level was maintained over a considerable

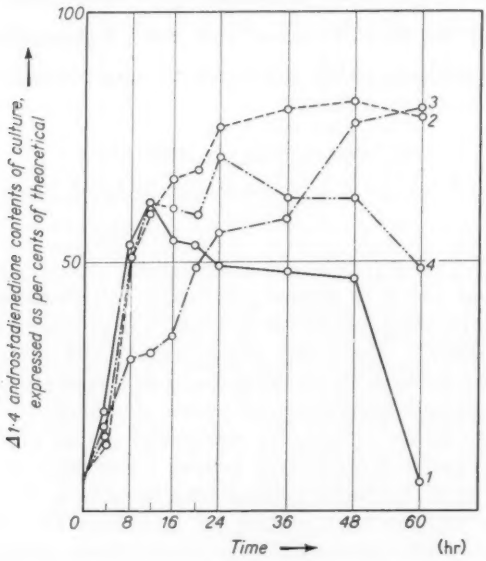

Fig. 1. Curve 1, 20 mg of progesterone in 1 ml acetone; curve 2, 20 mg of allopregnanedione in 2 ml acetone; curve 3, 20 mg of progesterone and 20 mg of allopregnanedione together; and curve 4, the sum of curves 1 and 2.

period. This latter circumstance is of decisive importance in the economical production of Δ1,4. Curves of a similar character could be observed when Δ4 and allopregnanedione were added together to the culture. The effect fails to appear, however, when pregnanedione is taken instead of allopregnanedione. When progesterone is added to the culture separately and the dosage of allopregnanedione follows in the 2nd, 4th, 6th, and 8th h, the effect is again to be observed. When allopregnanedione was first added to the culture and was followed at the above intervals by

the addition of progesterone, similar production curves for Δ1,4 were obtained. By changing the substrate ratio by dilution of the cultures, the mixed substrate effect became more pronounced with higher dilutions, so that in tenfold or 20-fold dilutions almost quantitative conversions could be obtained (Fig. 2). This

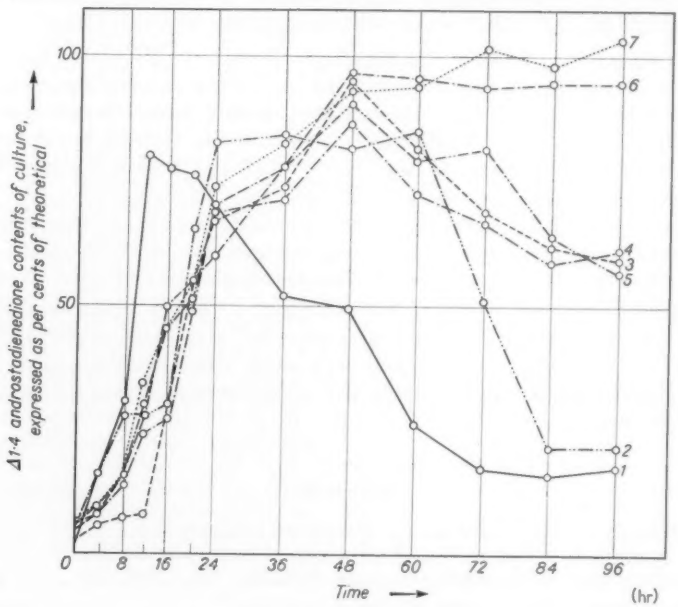

Fig. 2. Curve 1, 20 mg of progesterone in 1 ml acetone; curve 2, 20 mg of allopregnanedione in 2 ml acetone; curve 3, 20 mg of progesterone and 20 mg of allopregnanedione together; curve 4, the same as curve 3 but the culture was diluted twice with distilled water; curve 5, the same as curve 3 but the culture was diluted fivefold with distilled water; curve 6, the same as curve 3 but the culture was diluted tenfold with distilled water; and curve 7, the same as curve 3 but the culture was diluted 20-fold with distilled water.

favourable interaction of steroids could be shown also in Δ4-androstenedione–androstanedione–etiocholanedione mixtures. The addition of Δ4,3-ketosteroids mixed with 3-β-oxy-Δ5-steroids, e.g. with dehydroepiandrosterone or pregnenolone, fails to produce this effect. Neither can it be produced if these

3-β-oxysteroids are added to the culture together with saturated substrates, e.g. with allopregnanedione.

As a tentative explanation of this production increasing effect one might suggest that the saturated compounds are firmly bound to the enzyme surfaces and, in consequence, are less rapidly oxidized or over-oxidized. This property of the saturated compounds may be responsible for the prevention, or slowing down, of the oxidation of the unsaturated compounds to Δ1,4 and their further oxidation to Δ1-testololactone. This assumption is in agreement with the fact that in dilute culture media the effect is more pronounced. A similar phenomenon has been observed by Marcus and Talalay[3] in the case of isolated β-hydroxysteroid dehydrogenases. In their investigations, oestra–1, 3, 5,/10/–trien–3–ol, strongly adheres to the enzyme surfaces thus effectively blocking the activity of steroid dehydrogenase. In both experiments compounds belonging to the Δ4,3-keto and allo-series were found to be readily convertible substances. The 5 β-compounds, pregnanedione for instance, are converted in a lesser degree and in our experiments they did not show the effect which the 5 α-compounds exerted upon the oxidability of the Δ4,3-keto-structures.

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Book Reviews

Cholesterol by David Kritchevsky. John Wiley & Sons Inc., 1959. xi + 291 pp. 78/-.

The renewed interest manifest during the past two or three years in the biochemistry of cholesterol and its possible role in a number of diseases is reflected by the increasing number of books and symposia on this subject appearing in the past year. While most of these represent the collaborative effort of a number of contributors, the author of the present volume has attempted to cover the chemistry, biochemistry and pathology of cholesterol on his own. The result is a book which manages to compress an immense amount of factual material into small compass while remaining readable, but one which suffers from a lack of balance and authority in some parts at the expense of others. It would appear that the author never finally decided at which audience the book was aimed—the clinical scientist or biochemist.

The first section, on chemistry, is lucid and relatively complete with a particularly neat account of configurational problems and the reactions of cholesterol; also included in this section are the methods of synthesis of isotopically labelled cholesterols. The second section on biochemical aspects is not as well treated. The biosynthesis of cholesterol from small molecule precursors and the factors influencing this process are very adequately dealt with but the chapters on absorption and metabolism are somewhat summary, as is the section dealing with the possible conversion of cholesterol to steroid hormones.

The second half of the book concerns the role of cholesterol under physiological conditions and in various diseased states. The discussion on a possible causal relationship between cholesterol and atherosclerosis is very well presented and the literature reviewed covers the field from chemical and experimental pathology to medical statistics. The evidence that the disease is more probably linked to the overall fat intake rather than to any particular substance is well documented and convincingly argued. The final chapters on blood cholesterol and on methods of analysis are again most competent and complete. The variations in blood cholesterol in normal animals, under different dietary conditions and in many diseased states, are all minutely examined, particularly in relation to the human.

The book concludes with a series of appendices which are extremely useful in that they collect a great deal of scattered and unusual data on the physical properties of cholesterol and its derivatives, as well as distribution studies in different foods and species, into one place.

The whole work is one of considerable erudition and represents a fairly detailed account of the scope of existing knowledge on the subject. A measure of the task attempted is that this book, with 250 pages of text, contains over 2,000 relevant references.

A. L. GREENBAUM

Biochemical Preparations, Volume 6; Ed. Carl S. Vestling. New York, John Wiley & Sons, Inc.; London, Chapman & Hall, Ltd. IX and 105 pp. 42/–

Another volume in this useful series will be warmly welcomed by all concerned with biochemistry and biochemical techniques. Volume 6 well maintains the high standard of its predecessors and this is due in no little measure to the excellent technique of having each preparation tested by an entirely independent investigator. The worker who is unfamiliar with a particular type of preparation is thus assured that the description is adequate and that perseverance on his part is reasonably sure to result in success. This, unfortunately, is all too frequently not true of published descriptions in general.

One of the impressive aspects of the present volume is the wide variety of preparations among the 20 listed. The worker in the biochemical field must be prepared to obtain his varied and complex requirements as and where he can find them. Sometimes he may find a good biological source from which he may isolate the material (e.g. 2, 3-diphosphoglyceric acid from pig red blood cells), sometimes he may find a natural product from which he may readily prepare it (e.g. α-glycerophosphorylcholine from egg lecithin), but sometimes he may be forced to a lengthy synthesis (e.g. ^{14}C labelled tryptophan from labelled aniline). Whatever the method, he will have a good chance, and one which improves with the years, of finding it in this volume or its predecessors.

In addition to those mentioned above, noteworthy preparations in Volume 6 are crystalline cytochrome C from beef heart, and the very useful leucine amnopeptidase of pig kidney. The only criticism the reviewer has is to regret that in the deoxyribonucleic acid preparation no mention is made of Kirby's elegant and simple method. However, this may have been because it was published after this volume went to press.

E. M. Crook

Correction

Vol. I, No. 1, pages 43 and 44 : in the text on page 43 and in the captions to Figures 3 and 4 *Tribonema* should read *Asterionella* and vice versa.

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